이학박사학위논문

신경계에서의 mLLP 단백질의 역할 연구

Studies of the role of mLLP protein in the nervous system

2014년 8월

서울대학교 대학원

생명과학부

유 남 경

ABSTRACT

Studies of the role of mLLP protein in the nervous system

Nam-Kyung Yu

Department of biological sciences

The Graduate School

Seoul National University

Dynamic processes of neuronal morphogenesis during neural development require the complex regulation of a series of transcriptional programs, but there is much to be revealed to complete the whole picture of how gene expression is orchestrated for normal neuronal maturation. I found that a nuclear protein mLLP, a mouse homolog of ApLLP which is a transcription factor facilitating synaptic plasticity in *Aplysia* neurons, plays important roles in neuronal maturation. mLLP protein expression levels in the brain and cultured neurons are highest in the early developmental periods and decreases over the developmental time course. mLLP knockdown by expressing shRNA in the cultured neurons in the developmental phases impairs the dendritic growth and reduces the spine density as well as synaptic transmission. mLLP overexpression causes the opposite effect – increases in dendritic arborization, spine density, and synaptic transmission. These results suggest that mLLP is involved in the neural development.

mLLP protein has nuclear localization signals in N- and C-terminal regions, and it is localized to both nucleus and nucleolus. In nuclear extracts, mLLP protein interacts with various transcriptional regulators. Critically, it interacts with CTCF (CCCTC-binding factor) which is a multi-functioning transcription factor recently found to be important for neural development. Several CTCF downstream genes are downregulated by mLLP knockdown.

Interestingly, mLLP protein can be internalized into cells when extracellularly applied and this treatment increases the dendritic arborization, which is a similar effect of genetic overexpression of mLLP. Human homolog of LLP, which we named hLLP here, also has the transducible property.

Moreover, mLLP overexpression in the mouse hippocampal subregion dentate gyrus alters the adult neurogenesis and context discrimination learning, suggesting that mLLP could also modulate the neuronal function in the adult mouse brain.

.....

Key words: LLP, hippocampus, neural development, CTCF, PTD (protein transduction domain)

Contents

LIST OF FIGURES and TABLES

CHAPTER 1. INTRODUCTION

INTRODUCTION

Neural development is the most basic process for constructing the brain circuits that control animal behavior, cognition, and emotion (Tau and Peterson, 2009). Malformation of neural circuits during development can cause various brain disorders with symptoms such as mental retardation and autistic behaviors (Table. 1). It is important to understand the mechanisms of neural development to discover the treatment for the neurodevelopmental disorders or to find the ways to prevent the disorders. Moreover, the knowledge on how the brain circuits are initially generated will help understanding the mechanism of brain function and its evolution, which is one of the fundamental questions in neurobiology.

Neural development involves cell proliferation, differentiation, migration, and proper maturation of specified cells. Cells differentiated into the neuronal fates undergo dynamic structural growth (Cline, 2001). Mature neurons have an axon, dendrites, and spines, which are the complex specialized cell compartments for communicating with many other cells (Harris, 1994). The proper maturation of those neuronal structures is required for normal wiring of neural connections. Aberrant changes or malformation of these neuronal structures can cause neuropsychiatric disorders (Emoto, 2011; Kulkarni and Firestein, 2012) (Fig. 1).

Disorders considered involving neurodevelopmental problems	Neuropsychiatric symptoms
Attention deficit/hyperactivity disorder (ADHD)	Hyperactivity, Agitation, Impulsiveness, Being easily distracted
Autism spectrum disorders (ex: autism, Asperger syndrome, pervasive developmental disorder)	Impaired sociability, repetitive behaviors, language and speech problems
Fragile-X syndrome	Intellectual disability, autistic symptoms
Down syndrome	Intellectual disability, seizures
Rett syndrome	Impaired sociability, movement difficulties, Ataxia
Mendelsohnn's Syndrome	Impaired sociability, obsessivity, impaired motor skills, anxiety, schizotypy
Fetal alcohol spectrum disorder	Eye and ear abnormalities etc
Motor disorders (ex: Tourette syndrome, develop mental coordination disorder, etc)	Impaired motor function such as tics

Table 1. Neurodevelopmental disorders

Diseases thought to be caused by problems in neurodevelopmental processes and their neurological symptoms.

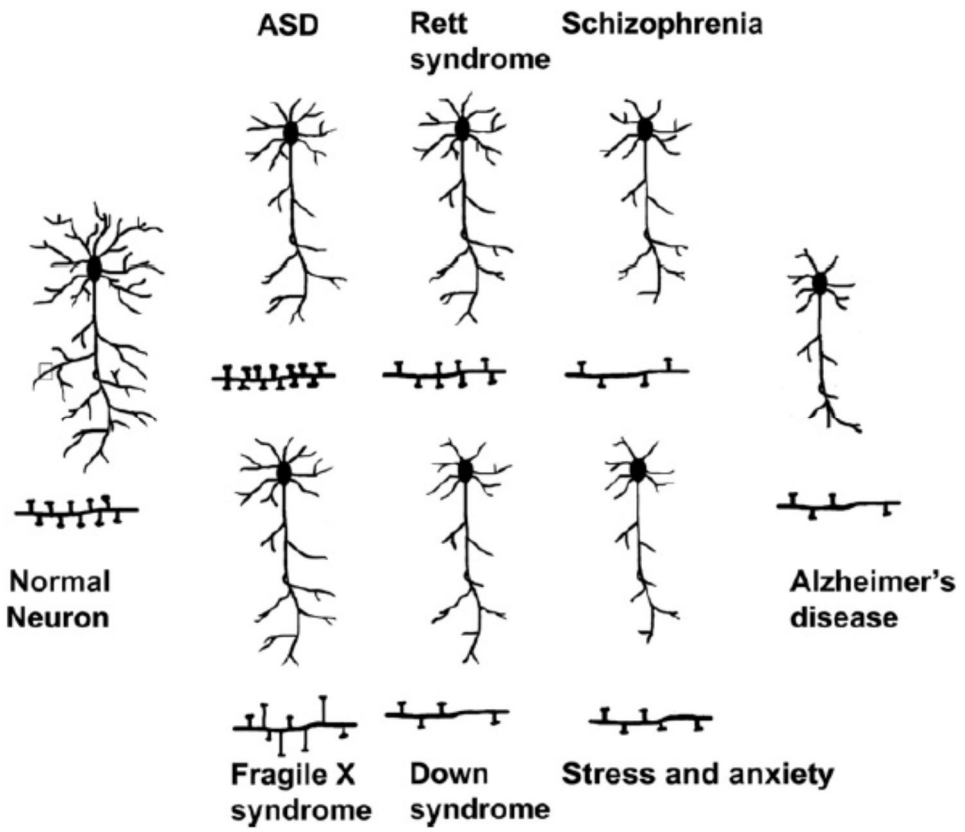


Figure 1. Abnormal neuronal structures associated with brain disorders (Kulkarni and Firestein, 2012)

Schematic representation of abnormal dendritic branching and growth patterns, spine morphology and density, reported in various brain disorders.

For these complex and dynamic changes during neural development, a series of transcriptional programs in the nucleus involving various transcription factors and chromatin remodeling factors should be meticulously orchestrated (de la Torre-Ubieta and Bonni, 2011; Ye et al., 2011). However, the detailed mechanism of transcriptional regulation during neural development is yet to be revealed (de la Torre-Ubieta and Bonni, 2011). Finding novel molecules regulating those transcriptional programs would provide an important building block for the complete picture of the transcriptional programs during neural development.

I have investigated a previously unknown nuclear protein mLLP and found that mLLP is important for neuronal morphogenesis during developmental phase. In my thesis, I firstly take a brief look on the neuronal structures and hippocampus, the main brain region I have focused on, and introduce previous studies on the invertebrate homologs of mLLP.

Structures of neurons

Neurons are the primary cell types transmitting information in the brain. They communicate with numerous other neurons in ordered ways, and these neural circuits in network are the basis of brain function to control animal behavior, cognition and emotion. For their function for conveying and processing information, neurons have unique structures distinct from other cell types (Kandel et al., 2000) (Fig. 2).

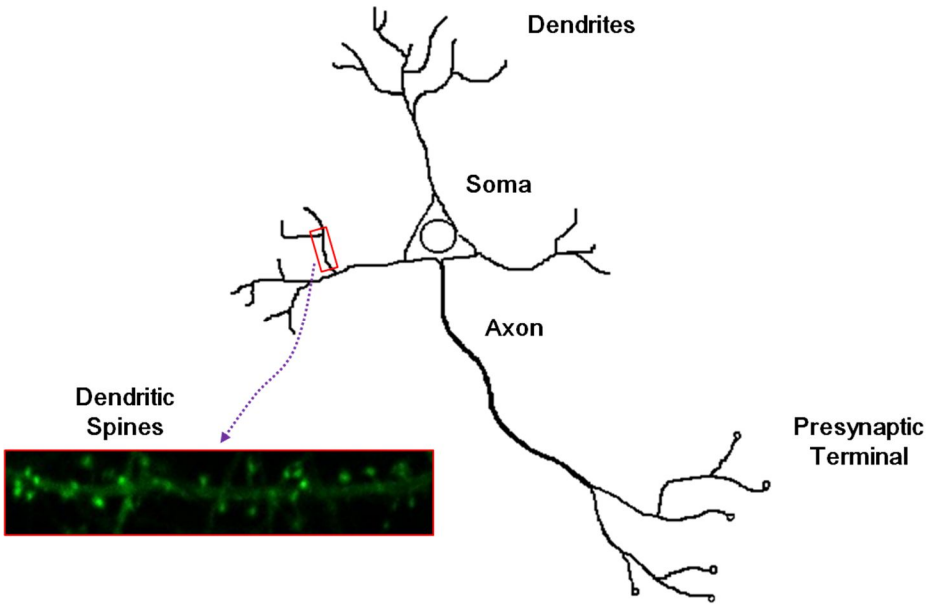


Figure 2. Typical structural components of neurons (modified from http://www2.estrellamountain.edu/faculty/farabee/biobk/biobooknerv.html)

A typical neuron has an axon and dendrites, the two types of neurites extending out from the soma. Axon and dendrites are the efferent and afferent signal transduction pathways respectively. They have certain branching patterns according to the cell types and functions, which is important for the information flow in the neural network circuit. Numerous small protrusions called dendritic spines are distributed along the dendrites. They are the major units receiving the excitatory inputs. Presynaptic terminals form synapse with the postsynaptic membrane at dendritic spines of other target neurons.

Axon is the output pathway of a neuron to transmit signals to other cells: mostly to neurons but muscles or glands in some cases. It is a long efferent projection that conveys electrical impulses away from the soma, the cell body. Axons make tight contacts with target cells forming junctions called synapses, where electrochemical signal transmission occurs. By branching and forming multiple synapses along the branch, an axon can innervate multiple cells and even multiple regions in the brain.

The other type of two kinds of neurites extending out from the neuronal soma is dendrite. Dendrites are mostly the input pathways of a neuron, receiving the signals via synapses from other upstream neurons. Not only are they important for collecting the inputs but also they are essential calculation units to determine the degree of signals to generate and send out to the downstream neurons. Dendrites are branched to form dendritic arborization and these branched segments can be used as a unit for the integrating signals received (O'Donnell and Sejnowski, 2014). Therefore, these arborization patterns and the outgrowth of dendrites are critical for determining the information transmission in the neural network.

Dendritic spines are small protrusions along the dendrites (Fig. 2). Spines are the specialized compartments receiving excitatory input via a synapse from a presynaptic terminal of the upstream neurons. They are distributed along the dendritic tree with density of two to more than ten spines per micrometer of dendrites in adult rodent brain. Spine head volumes are ranged from 0.001 μm^3 to 1 μm^3 and the neck length is shorter than 0.1 μm (Nimchinsky et al., 2002). Three major types of spines are known as thin, mushroom, or stubby spines, although recent super-resolution microscopy reveals that many stubby spines should in fact be categorized into mushroom type (Tønnesen et al., 2014). Typically,

neurotransmitter receptors and various structural and signaling components are densely located at the tip of the spines or synaptic membrane, which is called the postsynaptic density.

Hippocampus and memory

Synaptic strength can be either enhanced or decreased by strong or weak stimuli and maintained as changed. Stimulation and measurement can be conducted by electrophysiological recording. These phenomena of synaptic plasticity are thought to be the cellular models of how experiences change the brain circuits and are encoded in the brain. One of the brain regions extensively studied for this synaptic plasticity and its mechanism is hippocampus. Hippocampus is located in the medial temporal lobe in the brain and important for explicit memory formation, spatial and temporal information processing, and mood regulation (Fanselow and Dong, 2010). Dissociated hippocampal neuron culture is also the popular system to use for investigating the molecular mechanism of various properties of neurons (Kaech and Banker, 2006). In this research, I focused on the role of mLLP in the hippocampal neuron culture and adult hippocampus *in vivo*.

Hippocampus comprises subregions called CA1, CA2, CA3 and dentate gyrus (DG) (Witter, 2009). Circuits within the hippocampus and connections of each subregion with other external brain regions mediate different kinds of brain function (Langston et al., 2010). CA3-CA1 Schaffer collateral pathway is the most frequently examined region for recording synaptic plasticity. It is thought that associative memory is formed in this pathway. CA3 is implicated in pattern completion to mediate rapid retrieval of memory (Nakazawa et al., 2004). DG is

one of the few neurogenic niche where neurogenesis continues in the adult animals (Deng et al., 2010). DG-CA3 pathway is thought to be important for pattern separation (Leutgeb et al., 2007; McHugh et al., 2007; Sahay et al., 2011b; Yassa and Stark, 2011). Especially new born neurons in the dentate gyrus have been reported to contribute to pattern separation (Clelland et al., 2009; Creer et al., 2010; Niibori et al., 2012). Although hippocampal circuit and its roles have been extensively studied, still there are a lot to be revealed about the hippocampal circuitry, so that new findings about unknown cell types and related circuits are continuously being reported (Hitti and Siegelbaum, 2014; Kitamura et al., 2014).

Contextual fear associative memory is thought to be dependent on the hippocampus (Phillips and LeDoux, 1992). It is a standard behavioral paradigm to measure the degree of associative memory. Experimental subject is placed in a chamber, and kept to explore the context for several minutes, and then sudden electric footshock is given. The context is memorized associated with footshock, so that animals exhibit fear response measured as time freezing which is the state of lack of mobility except breathing. This robust fear memory is maintained throughout life and can be used as a model of posttrasumatic panic disorder (PTSD) (Grillon et al., 1996). Pattern separation is measured in this paradigm as context discrimination (McHugh et al., 2007). If animals are repeatedly exposed to the training context with shock and a similar but different context without shock, they gradually learn to discriminate the two contexts so that exhibit differential freezing behavior : show freezing more in the original context but less freezing in the no shock context. Adult neurogenesis in the dentate gyrus has been thought to be important for context discrimination learning, not critical for the initial contextual

fear memory formation (Sahay et al., 2011a).

Evolutionary conservation of LLP homolog (LLPh) protein sequences

LLPh family sequences are well conserved in animal species (Fig. 3A). Fig. 3B shows the LLPh amino acid sequences of various animal species as well as the properties of amino acids using the color codes. 46 of 130 amino acids of mLLP (~35.4 %) are conserved in Aplysia LLP sequences, and that 110 amino acids (~84.6 %) are identical with human LLP sequences. The N- and C-terminal enrichment of positive amino acids - arginines and lysines - is conserved, which suggests that this conserved charge distribution of amino acid sequences could be important for the LLPh function. No known functional domain is found in these amino acid sequences.

LAPS18 (Learning-Associated Protein of Slug with a molecular mass of 18 kDa)

The first identification of an LLPh family protein was reported in 2001 (Nakaya et al., 2001). The researchers found a gene encoding an 18 kDa protein among the genes induced by associative learning processes in *Lymax* nervous system, which they named LAPS18. mRNA and protein levels of LAPS18 were increased in the procerebrum(PC) where the odor information is thought to be processed after the odor-taste paired training. Interestingly, they reported that LAPS18 is a secretory protein and detected on the cell membrane surface. Dissociated PC cells plated on dish migrate and gather to form cell aggregates, which was facilitated by LAPS18 protein addition into the media but impaired by anti-LAPS18 IgG treatment.

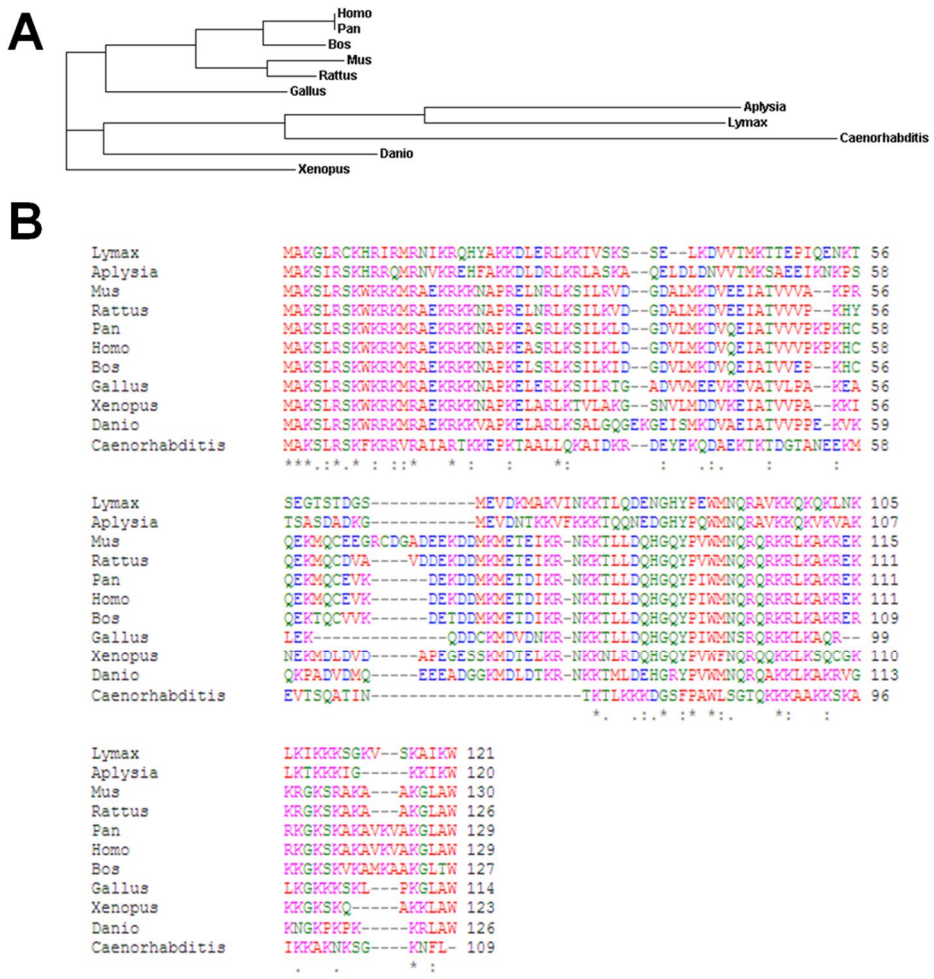


Figure 3. Amino acid sequences of LLPh proteins

(A) Phylogenetic tree of LLPh family proteins based on the amino acid sequences was drawn using ClustalW2.

(B) The amino acid sequence alignment of LLP proteins from various animal species using ClustalW2. Basic amino acids are indicated as magenta, acidic as blue, polar as green, and small or hydrophobic as red.

ApLLP (Aplysia LAPS18-Like Protein) and synaptic plasticity

Studies from our group showed that *Aplysia* homolog of LAPS18 was also upregulated by neural activity in *Aplysia* neurons (Kim et al., 2006). ApLLP upregulated ApC/EBP transcription to switch short-term facilitation to long-term facilitation so that the weak signal that normally induces only short-term facilitation could elicit long-term facilitation (Kim et al., 2006). Neuronal depolarization by elevated extracellular potassium level increases C/EBP, which lowers the threshold for inducing the long-term synaptic facilitation. This effect of depolarization was eliminated by injection of ApLLP antibody (Kim et al., 2006). Animals pretreated for noxious stimulus showed increased expression level of ApLLP in the nervous system, which suggested that this increased ApLLP will induce ApC/EBP which would facilitate the following learning. Indeed, this behavioral stimulus enhanced the memory formation so that weak training that induces only short term memory in control animals resulted in the long term memory in animals that experienced noxious stimulus previously (Kim et al., 2006).

ApLLP regulated ApC/EBP transcription as a nuclear/nucleolar transcription factor binding to asymmetric CRE element (CRE2) in the ApC/EBP promoter. It has nuclear localization signals in N- and C-terminal lysine-rich sequences, both of which were necessary and sufficient for nuclear/nucleolar localization (Kim et al., 2003).

Other LLP family proteins might also play an important role in the function of neural system. However, currently there is no report about mammalian homologues of ApLLP.

ApLLP and intrinsically unstructured proteins

One structural research on ApLLP showed that ApLLP is an intrinsically unstructured protein with no secondary or tertiary structures (Liu and Song, 2008), revealed by bioinformatic analysis and experiments using CD and NMR spectroscopy. An intrinsically unstructured protein (IUP) is a protein without an ordered three-dimensional structure (Dyson and Wright, 2005). Discovery of IUP had challenged the previous thought that the function of a protein depends on its 3D structure which is largely determined by its amino acid sequences. However, it has been found that there are a large number of disordered proteins the proportion of which reaches to about 30 % of eukaryotic proteins (Das and Mukhopadhyay, 2011).

Many IUPs or partially disordered amino acid sequences can adopt certain structures by interacting with other molecules and perform specific functions upon binding. In other cases, structure of IUP is still unfixed after binding to other molecules. ApLLP was shown to be the case as it was still unstructured with DNA binding (Liu and Song, 2008). This phenomena that IUP retains its disorder even upon binding with other molecules is called fuzziness and could be an important feature for the function of protein complexes (Fuxreiter, 2012).

IUPs or unstructured regions of proteins have been found to be functionally important. They are especially enriched in proteins involved in signaling (Iakoucheva et al., 2002), transcription (Dyson, 2012), and chromatin remodeling (Sandhu, 2009). Due to their flexibility, IUPs often can bind to multiple proteins, therefore acting as molecular hubs of protein-protein interaction network (Raychaudhuri et al., 2009). Therefore, protein-protein interaction and the

molecular or cellular contexts could be important determinants of the function of IUPs. As IUPs can interact with multiple partners, changes of the amount or conformation of IUPs unbalance the protein-protein interactions. Therefore, they are tightly regulated at level of transcription, translation, half-lives of mRNA and protein (Gsponer et al., 2008). Disruption of this fine control can be pathological (Das and Mukhopadhyay, 2011; Gsponer et al., 2008). Many oncogenes such p53 or BRCA have large unstructured regions which mediate the interaction with other molecules (Iakoucheva et al., 2002). α-Synuclein aggregation causing synucleopathies is thought to be due to its structural flexibility (Bertoncini et al., 2005).

Unstructured property of ApLLP could be an important clue for uncovering the molecular mechanism of how LLP family proteins work for regulating downstream genes.

Cell penetrating proteins

It has been thought that there are two modes of intercellular signaling by macromolecules across the cell membrane. Hydrophobic steroid molecules directly enter the cell and transmit signal by itself. However, hydrophilic proteins have been thought to bind the membrane receptors to transmit signals via intracellular second messenger and signaling pathways. This is because cell membrane is basically composed of phospholipid bilayer, which is a high energy barrier for hydrophilic macromolecules to penetrate. However, recently, cell penetrating proteins have been discovered (Prochiantz, 2000). The first one was Tat which is a HIV transactivator (Frankel and Pabo, 1988). The protein transduction domain

(PTD) called TAT is an 11 amino acid short peptide enriched with arginine and lysine and now being widely used to deliver macromolecules into cells (Heitz et al., 2009; Nagahara et al., 1998). Since Tat, other endogenous proteins of vertebrates especially among the transcription factors involved in development have been discovered to be cell penetrating proteins (Spatazza et al., 2013a). Internalized Engrailed-2 protein acted as an axon guidance molecule (Brunet et al., 2005). Interrupting the intercellular transfer of Pax6 led to the abnormal eye development (Di Lullo et al., 2011; Lesaffre et al., 2007). Otx2 signalling was suggested to be important for experience-dependent plasticity during postnatal development of visual cortex (Spatazza et al., 2013b; Sugiyama et al., 2008). These findings suggest there is a third mode of cell-to-cell signaling by macromolecules, which is the transduction of signaling protein itself (Prochiantz and Joliot, 2003). These proteins are also important in the aspects of application for therapeutics or biological research as protein transduction domains (PTD) artificially generated or derived from the endogenous transducible proteins have been used for macromolecule delivery into cells (Copolovici et al., 2014; Liu et al., 2014; Ramakrishna et al., 2014; Wang et al., 2014).

PURPOSE OF THIS STUDY

In chapter 2, I focus on the role of mLLP for neural development and behavior. I firstly show the developmental expression pattern of mLLP protein in the central nervous system. Then, to investigate the role of mLLP for neuronal development, overexpression or knockdown approaches in the primary neuron culture system were used. I present the results in multiple aspects of neuronal maturation: growth of axon, dendrites, spines and the following synaptic function. Related with function in the neurons, regulation of mLLP by neural activity was assessed. Furthermore, I show a behavioral consequence after mLLP overexpression in the mouse hippocampus.

In chapter 3, I investigate the molecular function of mLLP. Based on the previous studies about invertebrate homologs of LLP family and the high-throughput protein interactome analysis, I test the protein interaction of mLLP and the downstream molecules affected by mLLP knockdown. This might help to find out how mLLP regulates the neural development. In addition, I report a unique characteristic of mLLP, unstructured property and transducibility.

CHAPTER 2.

Role of mLLP in the neural development and behavior

INTRODUCTION

Gene expression pattern during mouse brain development can give us an important clue about the molecular mechanism of brain development and the function of a protein at specific developmental stage (Brumwell and Curran, 2006; Laeremans et al., 2013). Moreover, the gene expression pattern can be used to analyze and find out the pathogenic mechanism of disorders (Parikshak et al., 2013) . As the expression pattern of LLP in the mouse tissues is not known, I firstly analyzed the expression pattern of mLLP in the mouse tissues including brains to get a clue about the role of mLLP for the nervous system. The developmental expression pattern was assessed for the brain.

Primary neuron culture is prepared by dissociating the brain tissues and plating on the appropriately coated dishes (Kaech and Banker, 2006). Typically, neurons isolated from mice or embryos are attached onto the plate. Initially they have only cell bodies but a few protrusions. Day by day, neurites extend out from the cell body, an axon is elongated, and dendritic arborization and spines are actively developed. Synapses between the neurons are generated and synaptic transmission can be measured. This system is well known to recapitulate the development of neurons in the brain and share molecular mechanisms of synaptic transmission and plasticity. This monolayer culture system is easier to manipulate and analyze than the mouse brain so that it is one of the most frequently used experimental systems to investigate the molecular mechanism of nervous system.

During early days after the culture preparation, among the initially generated minor neurites from the soma, one longest neurite of a neuron becomes an axon

(Fig. 4). In later days, this thin and long axon sprouts further and is intermingled with branching and covering a large area making connections with many other cells. The other primary neurites become dendrites. They further outgrow, and secondary and tertiary dendrites are branched out to form arborization. Dendrites are typically shorter than axons and narrowing to the tip while axons have rather constant radii. Dendritic protrusions and spines begin to appear along the dendrites at around 10 days in vitro (DIV) and become denser day by day. Usually, the culture is thought to become mature at 14 DIV. As a model of neural development, I used this dissociated neuron culture system to investigate the role of mLLP for neuronal morphogenesis and maturation.

Then, to investigate the role of mLLP in the brain for behavior, I overexpressed mLLP in the mouse hippocampus DG and conducted experiments to test the contextual fear memory and context discrimination.

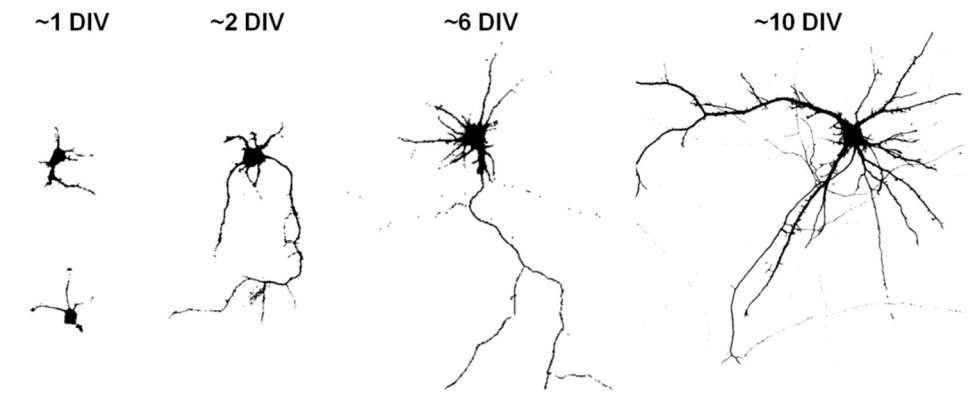


Figure 4. Phases of neuronal morphogenesis in dissociated neuron culture

After plating the dissociated neurons, among the minor neurites initially protruded, one neurite becomes longer and becomes axon. Dendrites grow and branch to form arborization, and dendritic spines begin to appear at around 10 DIV. Fluorescent images of ectopically expressed green fluorescent protein (GFP) were taken from the dissociated neuron culture expressing GFP at each DIV, and were converted to binary images for visualization using ImageJ.

EXPERIMENTAL PROCEDURES

Animals

All the experimental procedures were in accordance with the regulations of the Animal Care and Use Committee of Seoul National University. All the mice used were C57BL6/N from KOATEK company.

DNA constructs

mLLP cDNA was cloned by PCR with hippocampal cDNA of C57BL/6 as a template. It was again subcloned into the vector for expression in the mammalian cells (pcDNA3.1(+)-mLLP) or in the E.coli with hexahistidine tag attached at C-terminal (pET21a-mLLP). shRNA target sequences were GCCGAGAAGAGAAAGAAGA (shmLLP, KD) and . GAAAGAACGAGGAGAGACA (scrambled, SCR). The shRNA oligos were designed and inserted into the pSuper-GFP.neo vector following the product manual. shRNA-resistant mLLP sequence was generated by recombinant PCR and inserted into the pcDNA3.1(+) vector.

Antibodies and shRNA of mLLP

Antiserum against mLLP protein was generated in the rabbits injected with purified mLLP protein and further affinity-purified by attaching the antigen on nitrocellulose membrane. Antibody specificity was checked by immunoblotting the lysates from HEK293T cells expressing shRNA against mLLP with wild type mLLP or shRNA-resistant mutant mLLP. The intensity of mLLP band appearing at

about 18 kDa was apparently reduced by shRNA co-transfection, whereas shRNA-resistant mLLP signals were not affected (Fig. 5A). Knockdown of endogenous mLLP mRNA was confirmed by qRT-PCR (Fig. 5B) and protein level reduction was checked by immunoblotting (Fig. 5C). Together, these data show that the tools for research on the mLLP work well: shRNA effectively reduces mLLP mRNA and protein levels, the antibody specifically detects mLLP in the immunoblotting, and the shRNA-resistant mLLP can be expressed without disturbances by co-expression of the shRNA.

Adeno-associated virus (AAV) generation

The expression cassettes of shRNA from pSuper-GFP.neo-shmLLP or scrambled were inserted into AAV2 vector. For in vivo mLLP overexpression, mLLP cDNA was inserted into the expression cassette driven by EF1α promoter. AAV was generated by transfection of these vectors encoding transgenes and plasmids for AAV1 packaging into HEK293T cells (Choi et al., 2014). Follwing the purification by iodixanol gradient method from the supernatant media of transfected cells, the solution was concentrated and exchanged to PBS using Amicon Ultra-15. The titer of virus was measured as viral genome (vg) copy number calculated in comparison with the copy number of plasmids by real time PCR. 2-3 X 10^{12} vg/ml of AAV was generated.

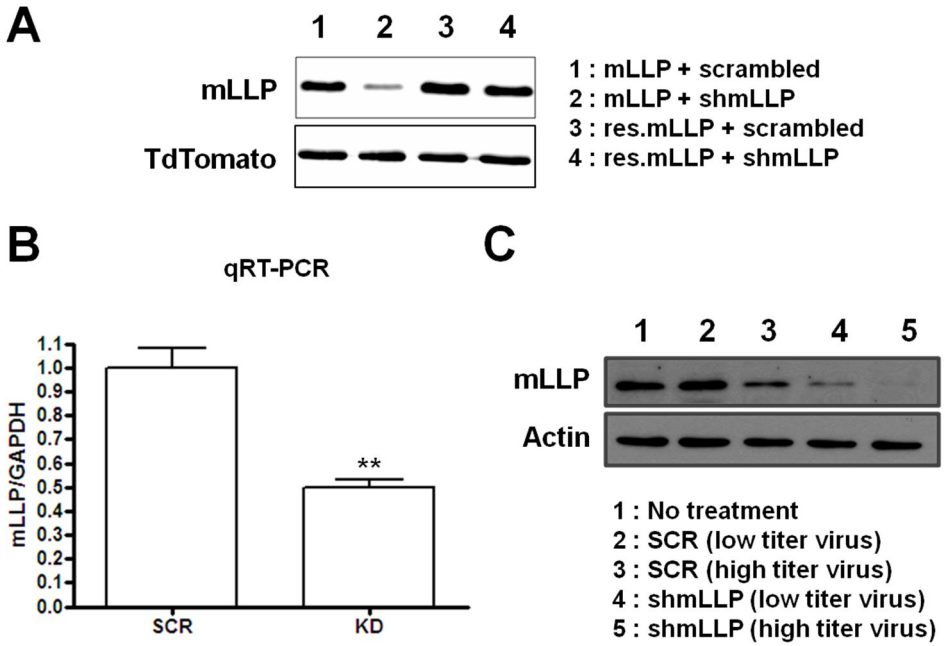


Figure 5. Validation for specificity of mLLP antibody, knockdown by shRNA against mLLP (shmLLP), and shRNA-resistant mLLP expressing construct

(A) Overexpressed mLLP protein in HEK293T cells was detected by the purified antibody as a band around 18 kD, which was diminished by co-expression of shRNA targeting mLLP. Signal of shmLLP-resistant res.mLLP was not affected by shmLLP co-transfection. To confirm the equal transfection efficiency, TdTomato was co-expressed and immunoblotted with RFP antibody. *(in collaboration with Juyoun Yoo)*

(B) qRT-PCR of endogenous mLLP mRNA in neurons infected with shmLLP-expressing adeno-associated virus (KD). mLLP mRNA was reduced to 50% compared to the group expressing control scrambled shRNA (SCR) (unpaired t-test, **p=0.0014, n=4 per group)

(C) Reduction of endogenous mLLP protein level by shmLLP knockdown.

Cell culture

Dissociated neuron culture was prepared following the general protocol (Kaech and Banker, 2006) with modifications. Hippocampi were dissected out from E17 embryos and dissociated mechanically after trypsin treatment. ~250,000 cells /cm^2 were plated onto the poly-D-lysine-coated plastic culture dishes or cover slips. Cells were maintained in the Neurobasal media supplemented with B27, glutamax, and penicillin/streptomycin. Transfection was performed using Lipofectamine2000 (Invitrogen) on the given days for each experiment.

HEK293T cells were maintained in DMEM/10%FBS, and transfection was performed by calcium phosphate precipitation method.

Immunoblotting

Scraped cultured cells, mouse whole brains (3-7 animals/age group), or various tissues were snap-frozen in liquid nitrogen and later lysed in RIPA buffer. The same amount of lysates were loaded and subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies (mLLP antibody (affinity purified from serum produced from the rabbit injected with purified mLLP protein), anti-GAPDH (Ambion, mouse), anti-c-fos (santa cruz, goat), anti-FLAG (Sigma, mouse)) overnight at ~4℃ and then with HRP-conjugated secondary antibodies for 30 min at room temperature. Chemiluminescent signals were detected with HRP substrates (Millipore, WBKLS0100) using ChemiDoc system or developing solutions, and the band intensities were quantified using ImageJ software.

qRT-PCR

RNA was extracted using TRIZOL from the primary neuron culture infected with AAV. Then, it was reverse transcribed using SuperScriptIII reverse trascriptase and random hexamers. Real-time PCR was performed using SYBR premix Ex TaqII. Primer sequence is as follows. mLLP : GAG ATA GCA ACC GTG GTG GT (forward), GCC TCT GGT TCA TCC ACA CT (reverse).

Image analysis

For dendrite analysis, GFP fluorescence images from the transfected neurons were taken by epifluorescence microscope with 10X or 20X objective lens. NeuronJ plugin of ImageJ was used for tracing and measuring the neurite length.

For spine analysis, transfected neurons were imaged with 40X objective lens and Z-stack mode in confocal microscope (LSM700). Viewed in Zen (Zeiss) software, spine numbers were counted for three 50 μm random dendritic segments and averaged per neuron.

Nine to ten neurons per group were analyzed from single set of culture, which was repeated for three independent cultures. Imaging, tracing and counting were performed being blind to the group.

Immunocytochemistry for synaptophysin puncta analysis

Cells were fixed with 4% paraformaldehyde / 4% sucrose in PBS. These cells were permeabilized with 0.1% Triton X-100, 0.1% BSA in PBS (PBT) and then blocked with 0.08% Triton X-100, 2% BSA in PBS. Antibodies were incubated in blocking solution. Primary antibodies (anti-synaptophysin, rabbit, santa cruz; anti-PSD95,

ABR, mouse) were incubated o/n at 4℃ and washed with PBT. Secondary antibodies conjugated with fluorescence dyes were incubated at room temperature for 2 hrs and washed with PBT. Samples were mounted on Vectashield with DAPI (VectorLab) and images were taken using confocal microscopy (LSM700) choosing the planes with most clear PSD95 puncta along the z-axis. For the synaptophysin images, neurites were chosen randomly and the immunopositive puncta density on the neurite was analyzed using ImageJ after consistent thresholding.

Electrophysiological recordings.

For whole-cell patch-clamp recordings, Cultured hippocampal neurons (DIV 10~11) were patched and kept in -70mV using a Multiclamp 700B amplifier and pClamp 10.2 software (Molecular Devices). Data were collected for 5min and digitized at 10 kHz with a 2 kHz low-pass filter using Digidata 1440 16-bit A/D converter (Axon instruments). The recording pipettes (3~5 MΩ) were filled with internal solution containing 145 K-Gluconate, 5mM NaCl, 0.2mM EGTA, 10mM HEPES, 2mM MgATP, 0.1mM Na3GTP, 1mM MgCl2 (pH 7.2 with KOH, 280~290 mOsm). The bath solution contained 124mM NaCl, 2.5mM KCl, 1mM NaH2PO4, 25mM NaHCO3, 10mM Glucose, 2mM CaCl2, 2mM MgSO4. For mEPSC recording, Picrotoxin (100 μM) and Tetrodotoxin (1 μM) were added to the bath solution to block GABAA receptor mediated current and to block evoked synaptic responses, respectively. The bath solution was oxygenated with 95% Co2, 5% O2 mix gas and perfused 2 ml/min at 25~26°C. Series resistances were carefully monitored and recordings were not used if it changed significantly (>20%) or reached 20MΩ.

Cells that required more 200pA of hold current to maintain -70mV were excluded from the dataset. Data were analyzed using Clampfit 10.4 (Molecular Devices) with template match threshold of 4.

Surgery

C57BL/6 male mice of 8 weeks old were anesthetized with ketamine/xylazine i.p injection, and placed on the streotaxic. AAV 0.5 μL was injected into the hippocampal DG bilaterally with coordinates of AP -1.9 mm, ML ±1.5 mm, DV -2.2 mm. Automatic injection pump was used to inject the virus with speed of 6 μL/hr, and 10 min was waited before the removal of the injection needle.

Behavior

Mice were daily handled for 4 days before contextual fear conditioning. Contextual fear conditioning was performed in Coulbourn fear conditioning chamber unit using Freezeframe software. Mice were stabilized at least 30 min in the testing room before the daily experiment started. For contextual fear conditioning, mice were placed in the chamber and stayed for 3 min before the 0.6 mA electric footshock for 2 seconds was given. After 15 seconds, mice were put back into their home cages. According to the order diagram in the figure, each mouse was exposed to the training context and a different context without shock every day. Shock was given in the training context but not given in the different context. Fear conditioning chamber of Med Associates Inc. was used as the no shock context. The mice with no virus expression were excluded from the analysis.

RESULTS

Developmental expression pattern of mLLP in the brain and primary neuron culture

The developmental expression pattern of mLLP protein was assessed in the whole brain lysates of mouse embryos or mice with age of postnatal day 1, 5, 10, 20, 56. Immunoblotting the lysates with mLLP antibody showed stronger expression of mLLP in the early developmental phase and sharp decline until the adulthood (Fig. 6A). The same pattern was recapitulated in the dissociated mouse hippocampal neuron culture (Fig. 6B). mLLP protein levels were much higher in young neurons than in mature neurons. The transcriptome data in Allen Brain Atlas database showed that human and monkey mRNA levels of LLPh also tended to developmentally decrease in most brain regions. An example is shown in Fig. 7A-B.

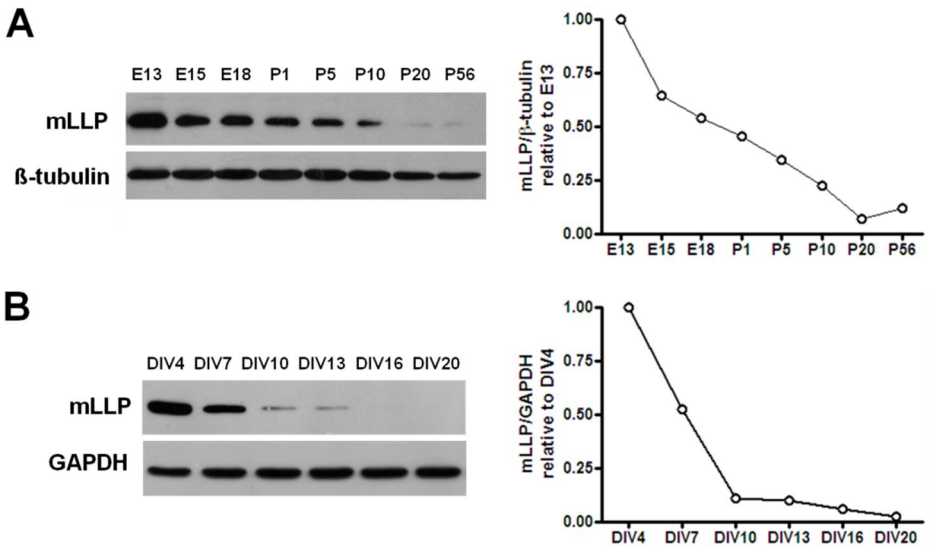


Figure 6. mLLP protein level in the brain decreases throughout development

(A) Left, Immunoblotting of whole brain lysates from mice of various ages. Right, quantification. (B) Left, Immunoblotting of lysates from dissociated mouse hippocampal neuron culture of various stages. Right, quantification.

(in collaboration with Seohee Ahn)

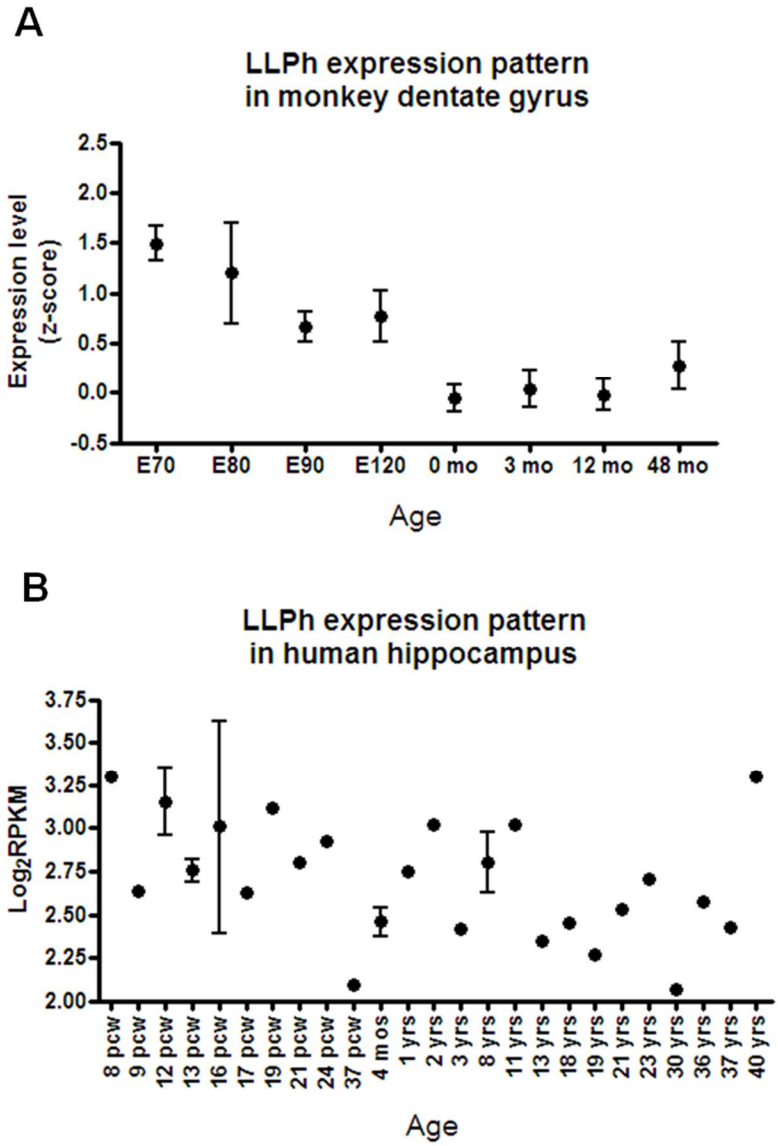


Figure 7. Developmental expression pattern of LLPh in the primate brains (data from Allen Brain Atlas)

An example of the developmental monkey (A) or human (B) transcriptome data from Allen Brain Atlas (http://www.brain-map.org/) was represented as a graph along the developmental time course.

Effects of mLLP knockdown or overexpression on the neurite growth

As mLLP is expressed with higher level in the brain at earlier developmental stages, I hypothesized that mLLP might be an important regulator of neural development. I examined whether mLLP is required for the development of neuronal morphology in the dissociated neuron culture where the neuronal morphogenesis phases are observed in about two weeks after plating onto the culture dish. Four experimental groups were examined to see the effects of both reducing and increasing the mLLP level (Fig. 8A). The neuron culture was transfected with shRNA-expressing plasmids or scrambled control that co-express EGFP, together with plasmids encoding shRNA-resistant mLLP or control vectors. The rescue group was included to see whether there is an off-target effect of shRNA. Firstly, I focused on the axonal outgrowth at early stage. During early phase of neuronal maturation, axon extends longer than dendrites before the active dendritic growths. Among the neurites extending in this early maturation phase, the longest neurite becomes an axon. I transfected plasmids into the neuron culture at 1 DIV and fixed the cells next day. Co-expressed GFP images were taken by confocal microscope, and the length of the longest neurite per cell that becomes the axon of the cell was measured. (Fig. 8B). The axonal length appeared not affected by mLLP overexpression or knockdown in this experimental condition (one way ANOVA, $p=0.1621$, n=37-45 cells per group) (Fig. 8C).

Then, to see whether mLLP regulates dendritic growth, knockdown or rescue plasmids were expressed from 3 DIV to 6 DIV, and the primary dendritic lengths were measured for each transfected neurons and analyzed. Knockdown of mLLP increased the proportion of short primary neurites (< 30 μm) per cell (one-way

ANOVA and post hoc Tukey's multiple comparison test, *$p<0.05$, ***$p<0.001$) (Fig 9A). When mLLP protein expression was rescued with shRNA-resistant mLLP, the number of short primary neurites per cell was recovered to the control level. Overall distribution of primary neurite lengths taken from neurons examined also exhibited the shortening of neurites by mLLP knockdown and enhancement by mLLP overepxression (Fig 9B). These results suggest that mLLP regulates dendritic growth during neuronal maturation.

A

CTL (control)	OE (overexpression)	KD (knockdown)	Rescue (of protein)
1. Scrambled shRNA + GFP 2. Vector control	1. Scrambled shRNA + GFP 2. shRNA-resistant mLLP	1. mLLP shRNA + GFP 2. Vector control	1. mLLP shRNA+ GFP 2. shRNA-resistant mLLP

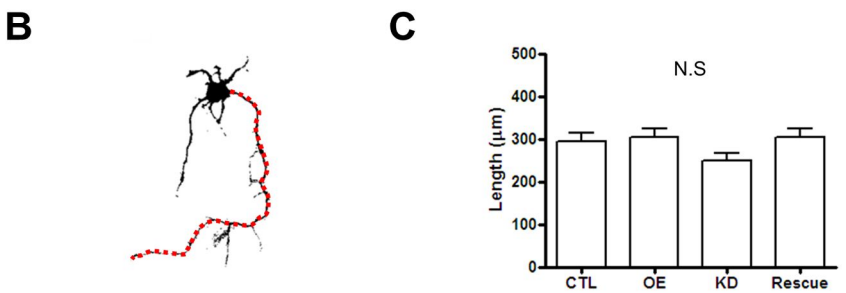


Figure 8. mLLP overexpression or knockdown does not affect axon outgrowth

(A) Experimental groups examined to see the effects of both reducing and increasing the mLLP level. The neuron culture was transfected with shRNA-expressing plasmids or scrambled control that co-express EGFP together with shRNA-resistant mLLP-expressing plasmids or control plasmids. The rescue group was included to see whether there is an off-target effect of shRNA.

(B) After transfection at 1 DIV in dissociated hippocampal neuron culture, longest neurite length was measured for each neurons at 2 DIV to examine the axon outgrowth.

(C) Axonal lengths were not statistically different among the groups. *(in collaboration with Somi Kim)*

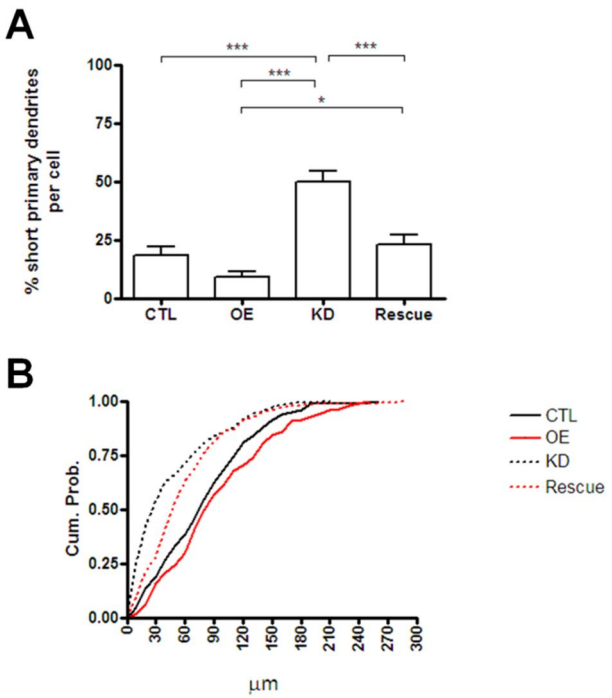


Figure 9. mLLP regulates primary dendrite outgrowth

(A) Ratio of primary dendrites shorter than 30 μm was increased by mLLP knockdown, which was recovered by co-expression of res.mLLP (n=29-30 cells per group)

(B) Total distribution of primary dendrite lengths of the cells analyzed.

Effects of mLLP knockdown or overexpression on the spine density

To examine whether mLLP knockdown would alter synaptogenesis, shRNA-expressing AAV was infected into the neuron culture, and the culture at 8 DIV was immunostained against presynaptic marker synaptophysin (Fig. 10A). Image analysis showed that synaptic density of infected neurons is decreased by mLLP knockdown (unpaired t-test, $p=0.015$, $n=40$, 55 cells) (Fig. 10B), suggesting the role of mLLP for synaptogenesis. However, as this experimental condition affected almost all the neurons in a dish, it could not distinguish whether this effect was due to the presynaptic or postsynaptic mechanism.

As dendritic and spine growth often involve common molecular mechanisms, we hypothesized that mLLP could act in the postsynaptic neurons and regulate the synaptic density through affecting the spinogenesis. By transfecting plasmids expressing GFP which sparsely labels cells in a dish, spine density of transfected neurons was measured. Spine density was significantly reduced by knockdown of mLLP from 6 DIV to 10 DIV, which was partially recovered by shRNA-resistant mLLP (one-way ANOVA and post hoc Newmann Keuls multiple comparison test, $*p<0.05$, $**p<0.01$, $***p<0.001$) (Fig. 11A). Overexpression of mLLP increased the spine density (Figure 2C, $*p=0.0148$, unpaired t-test, $n=30$, 29 cells) (Fig.11B), supporting the regulation of spine structure by mLLP.

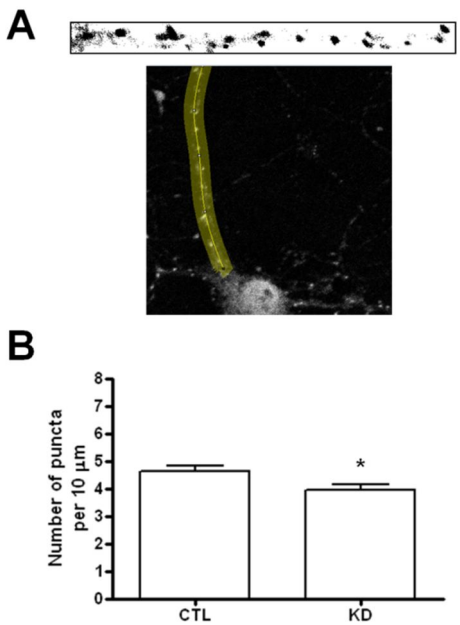


Figure 10. mLLP knockdown decreases the synaptic density

(A) Anti-synaptophysin immunostaining image and its analysis. Using ImageJ, a random primary neurite was chosen and linearized to measure the particle density analysis.

(B) The synaptophysin puncta density was reduced in mLLP knockdown group.

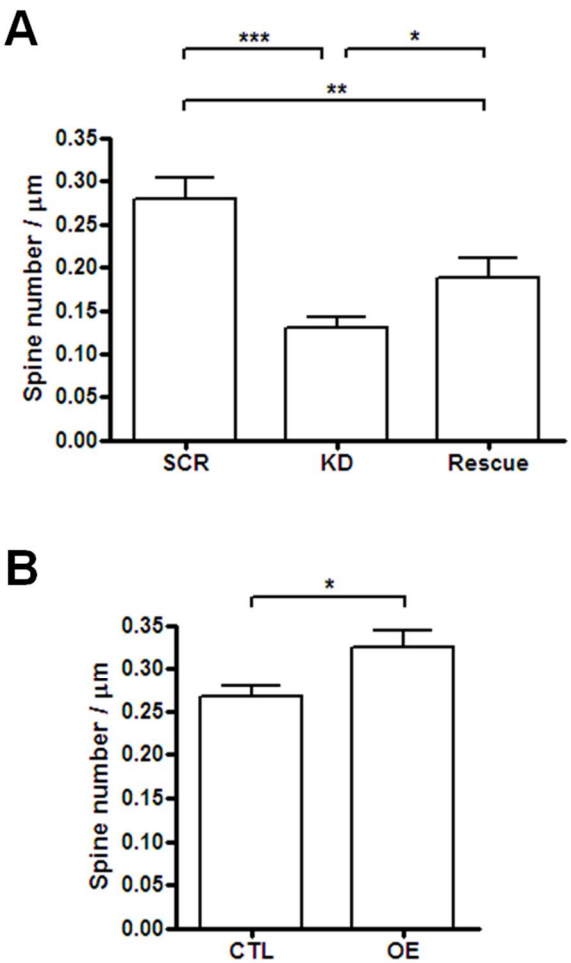


Figure 11. mLLP regulates spine density *(in collaboration with Somi Kim)*

(A) mLLP knockdown decreases the spine density at 10 DIV. The effect of mLLP shRNA was partially reversed by co-expression of mLLP the sequence of which is resistant to shRNA.

(B) mLLP overexpression increases the spine.

Effects of mLLP knockdown or overexpression on synaptic transmission

Furthermore, we examined whether mLLP could regulate synaptic transmission. Miniature excitatory postsynaptic current (mEPSC) is a measurement of postsynaptic response elicited by spontaneous single vesicle release of glutamate from presynaptic terminal (Fig. 12A). mEPSC amplitude was not significantly altered by mLLP overexpression but mLLP knockdown decreased the mEPSC amplitude (Fig. 12C). mLLP overexpression increased the mEPSC frequency, consistent with the increase of spine density (One-way ANOVA with post hoc Tukey's multiple comparison test, *$p<0.05$, **$p<0.01$, ***$p<0.001$) (Fig 12B). These data show that mLLP also affects synaptic transmission possibly through regulating the spinogenesis.

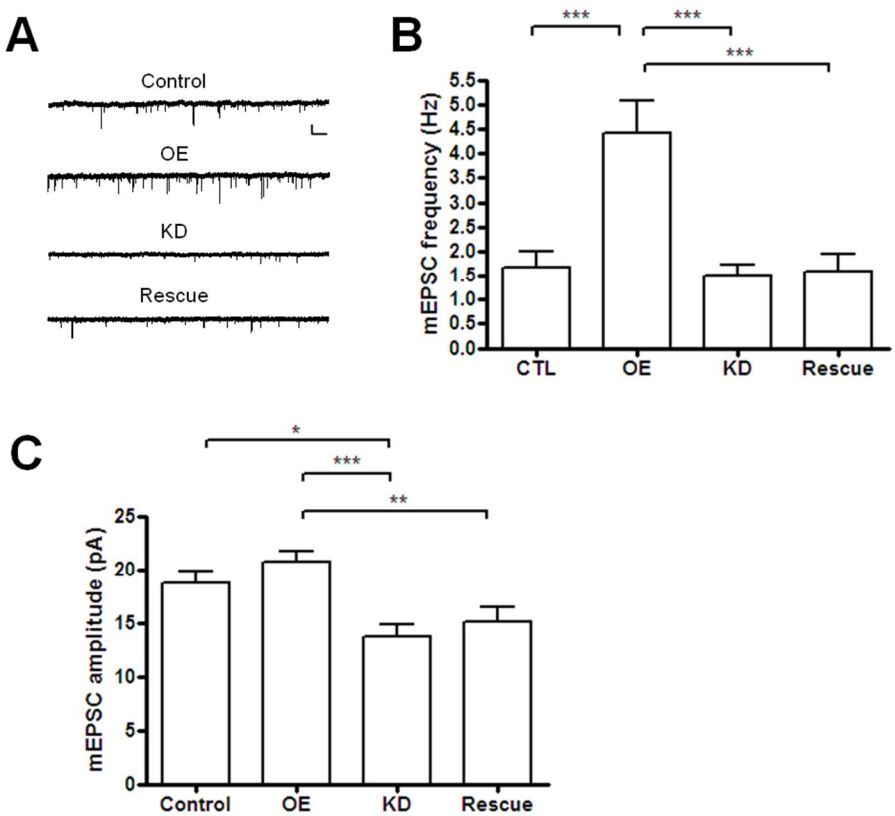


Figure 12. mLLP regulates synaptic transmission *(in collaboration with Jaehoon Shim)*

(A) Sample recording traces of mEPSC recorded in dissociated hippocampal neuron culture transfected with plasmids for expressing shRNA against mLLP or scrambled control and plasmids overexpressing shRNA-resistant mLLP or its control plasmids. Scale bar, x=500 ms, y=20 pA.

(B-C) mEPSC frequency and amplitude show that mLLP overexpression and knockdown affect synaptic transmission (n=12-13 cells).

Neural activity downregulates mLLP

Neuritogenesis and spinogenesis are thought to be regulated by neural activity during development (Zhang and Poo, 2001). mLLP might be the mediator of controlling neuronal morphogenesis by neural activity. As ApLLP was induced by neuronal depolarization (Kim et al., 2006) and LAPS18 was upregulated by associative learning event (Nakaya et al., 2001), it was predicted that mLLP would also be induced by neural activity. Dissociated cultured mouse neurons were exposed to the stimulation of high concentration of extracellular potassium (40 mM KCl), and the lysates were subjected to the immunoblotting. Unexpectedly, mLLP protein level was rather reduced by neural activity (Fig 13). Reduction of mLLP by neural activity was also shown in qRT-PCR suggesting the RNA level regulation (Fig. 14A-B). However, there might be also a posttranslational regulatory mechanism for downregulating mLLP by neural activity (Fig. 15). *In vitro* calpain reaction showed that mLLP is a substrate of calpain, which is an activity- and calcium-dependent preotease (Zadran et al., 2010). These results suggest there is a different upstream regulatory mechanism of mLLP and invertebrate LLP homologs.

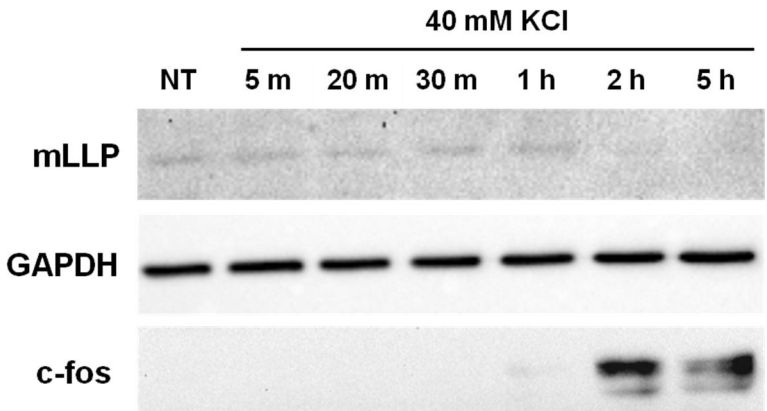


Figure 13. Elevated neural activity decreases mLLP protein level

c-fos, which is a neuronal activity marker, was robustly induced by high potassium treatment. However, in the same lysates, mLLP protein appeared to be reduced (NT: No Treatment, m: minutes, h: hours of KCl treatment duration).

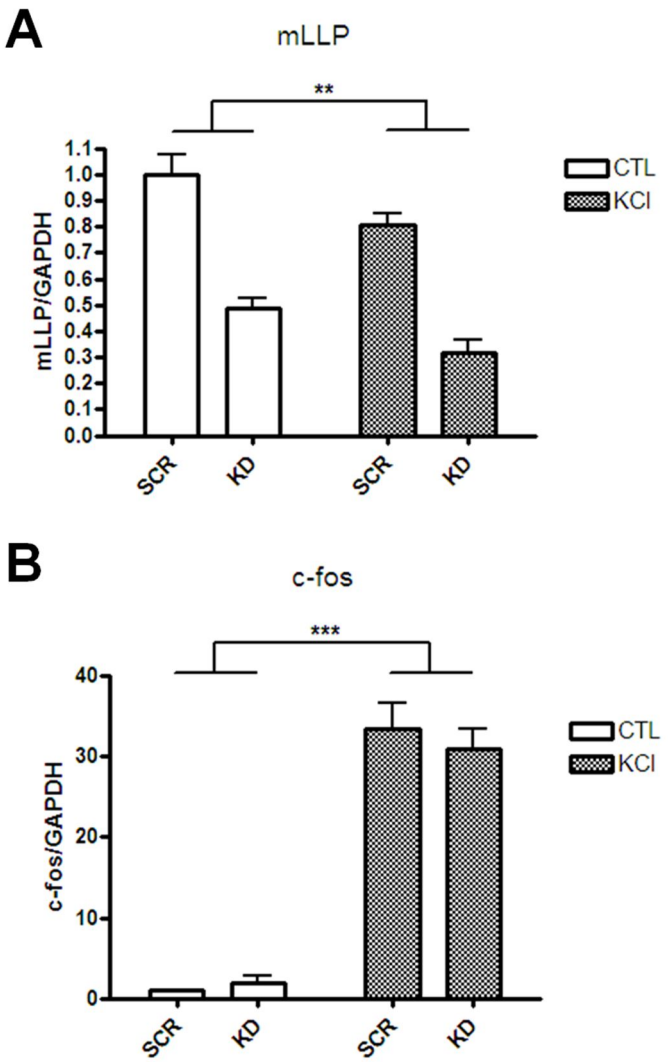


Figure 14. Elevated neural activity decreases mRNA level of mLLP

(A) mLLP mRNA level was reduced by ~20 % by KCl treatment to the neuron culture for 2 hours. There were significant effects of KCl (**p=0.0084) and shRNA ($p<0.0001$) (Two-way ANOVA).

(B) In the sample prepared in (A), c-fos was highly induced by KCl treatment indicating the elevated neural activity (***$p<0.0001$).

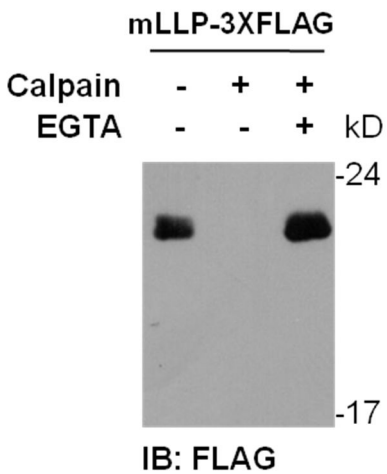


Figure 15. mLLP proteolysis by calpain

Lysates of HEK293T cells expressing mLLP-3XFLAG were subjected to calpain reaction with or without EGTA, calcium chelator. Calpain-mediated proteolysis of mLLP was dependent on calcium *(in collaboration with Jun-Hyeok Choi).*

mLLP overexpression in DG alters context discrimination behavior

As mLLP regulates synaptic transmission, mLLP could play a role in controlling animal behavior *in vivo*. Mouse hippocampus comprises multiple subregions: CA1, CA2, CA3, and DG. Among them, DG, which is one of the few neurogenic niches in the adult brain, was targeted for injecting AAV overexpressing mLLP with GFP or GFP alone as a control (Fig. 16). After a month to wait for the AAV viral gene expression, behavioral experiments were performed.

In contextual fear conditioning paradigm, mice received a single electric footshock upon exposure to a novel context. Robust context-shock associative fear memory was formed, which was measured as high percentage of time spent freezing (Fig. 17C-D). The strength of memory tested on the next day of training was not different between the experimental and control groups (unpaired t-test, p=0.9182).

Next, context discrimination behavior was tested as the targeted region DG has been implicated in context discrimination learning which is related with pattern separation (McHugh et al., 2007; Sahay et al., 2011a). I followed the experimental protocol (Fig. 17A) used in a previous study (Sahay et al., 2011a). Initially, mice exhibit freezing indistinguishably in both contexts, but as discriminative learning proceeds, they gradually show higher freezing in the training context than in the "no shock context". Compared with control mice, mLLP overexpressing mice discriminated the two contexts more slowly (two-way RM ANOVA, group effect *p=0.0190) (Fig. 17B). Overall, during training days, control mice showed significant difference in the two contexts (two-way RM ANOVA, context effect *p=0.0206) (Fig. 17C) but mLLP overexpression group did not (context effect

p=0.5132) (Fig. 17D).

To examine whether neurogenesis in the DG is correlated with the context discrimination performances, immunostaining of hippocampal tissues from mice used for the behavioral experiments was performed using the Ki-67 antibody, a proliferating cell marker. Inconsistent with previous reports showing that DG neurogenesis is required for and facilitates pattern separation, cell proliferation in DG was rather enhanced by mLLP overexpression which had impaired context discrimination (Fig. 18A). Since the viral gene expression was primarily strong in the dentate gyrus granule cells but not in the proliferating cells (Fig. 18B), the effect of mLLP overexpression on the neurogenesis does not seem to be cell-autonomous.

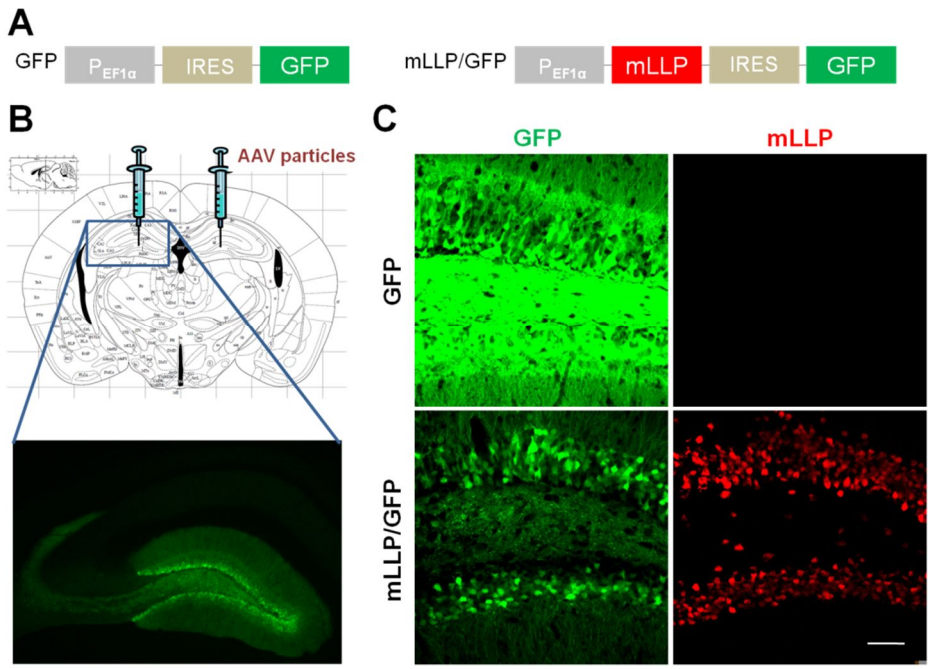


Figure 16. mLLP overexpression in the mouse hippocampus

(A) AAV overexpressing either mLLP with GFP or GFP alone as a control was injected into the mouse hippocampus dentate gyrus (B).

(C) Immunostaining with mLLP antibody confirmed that mLLP is robustly overexpressed in this experimental condition. Scale bar, 50 μm.

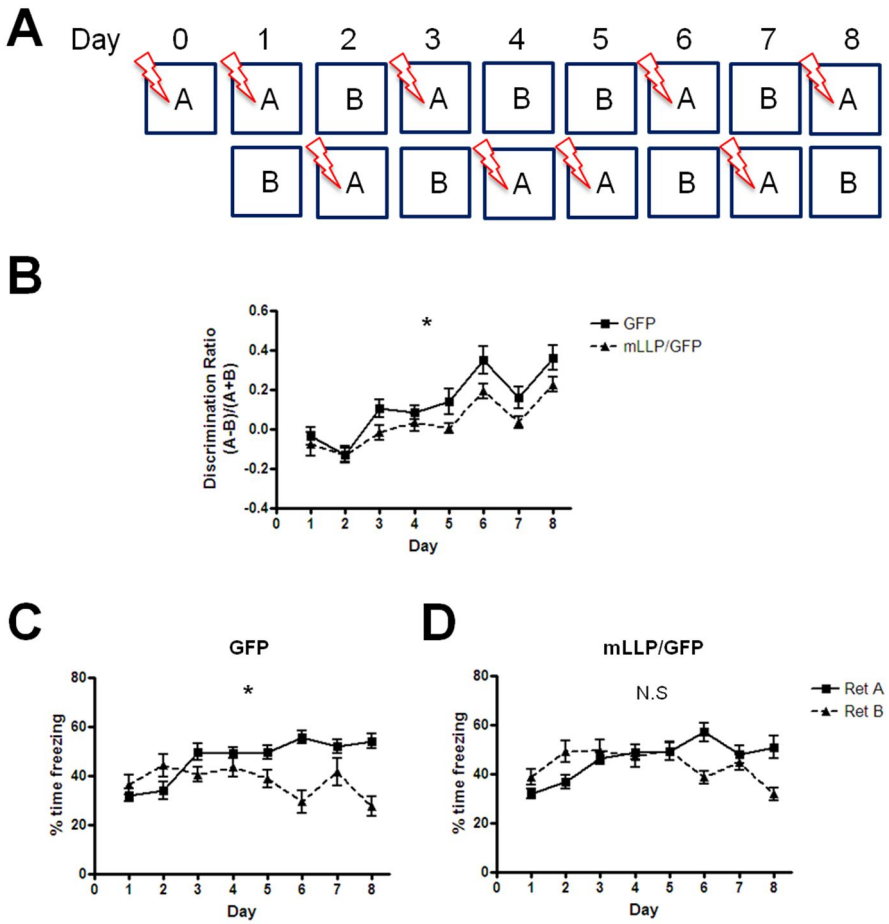


Figure 17. mLLP overexpression in the mouse dentate gyrus alters the context discrimination learning

(A) Context discrimination learning protocol. Following initial contextual fear conditioning in the context A, mice were daily exposed to the re-conditioning in the original context A or to the similar but different context B without footshock. The daily order of exposure to the contexts is shown. (B) Context discrimination was assessed by discrimination ratio : [(% time freezing in context A)-(% time freezing in context B)]/[(% time freezing in context A)+(% time freezing in context B)] n=12, 14 animals per group. (C-D) Percentage time of freezing to each context on each day is shown for each group.

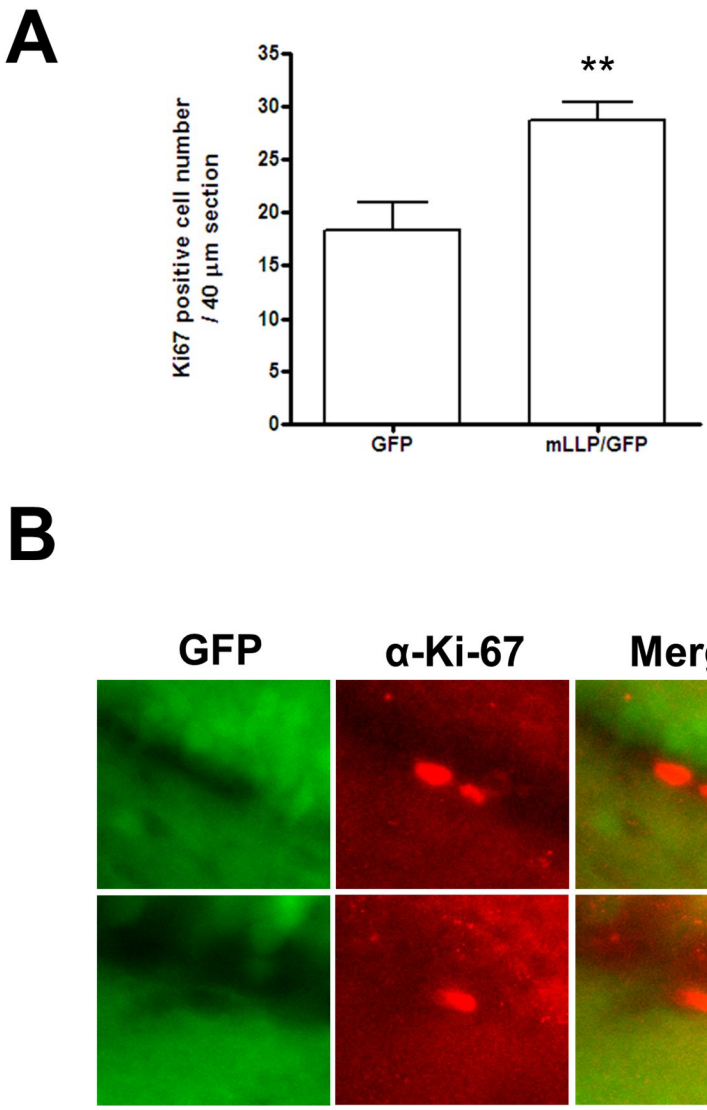


Figure 18. mLLP overexpression enhances cell proliferation in the DG, which seems to be a non-cell-autonomous effect *(in collaboration with Somi Kim)*

(A) Number of Ki67 positive proliferating cells in the dentate gyrus was increased by mLLP overexpression. (B) No Ki67 positive cell showed apparent GFP expression in the dentate gyrus of mice injected with the AAV.

DISCUSSION

The present results show that mLLP is a developmentally regulated protein in the central nervous system and that it regulates neural development in terms of dendritic growth, spinogenesis, and synaptic transmission. Neural activity downregulates mLLP mRNA and protein. In addition, mLLP overexpression in the dentate gyrus impairs pattern separation, giving the initial clue to resolve the role of mLLP in the nervous system.

According to the RNA-seq database (BrainSpan), the mRNA expression pattern in the developing human brain also shows the pattern similar to our results. Expression level of hLLP (human homolog of LLP) is shown to decrease throughout development in most of the brain regions. As amino acid sequence is well conserved in humans and mice (~78%), LLP protein might have a similar role in human brain development as well.

Recent bioinformatic analyses have shown that some co-expression modules of autism spectrum disorder (ASD) genes are more strongly expressed in the earlier developmental periods and enriched with transcriptional regulators (Parikshak et al., 2013). Many rare de novo variant genes were included in these modules, implicating that there should be more yet unknown variants. These might include LLPh, as it is a putative transcriptional regulator that follows the similar developmental expression pattern with the modules and affects neural development.

This study was performed only in the primary neuron culture system. It provides a clue for the function of mLLP in the neural development as the neuron culture system is thought to recapitulate the brain developmental processes. However, *in*

vivo approach such as studies with knockout mice would be required to investigate whether LLP is indeed important for neural development and how the deficiency affects the brain development.

Membrane-depolarizing treatment for 2 hrs downregulated mLLP at mRNA and protein levels. This reduction might be important for homeostatic plasticity, as hours of neural activity could initiate homeostatic process for downregulating synaptic strength or excitability. Homeostatic plasticity is considered to be important for neural development, and mLLP might be one of the molecules mediating the homeostatic regulation and neuronal morphogenesis. Whether reduction of mLLP protein by neural activity involves calpain activity would be another future question as calpain is also important for dendrite morphogenesis (Amini et al., 2013).

It is also unknown whether mLLP affects the spine formation or elimination and whether it regulates dendrite growth or pruning. Dendrite pruning or spine elimination actively occurs during development, and the possibility whether mLLP is important for the positive or negative regulation is an open question.

Impaired contextual discrimination performance and increased cell proliferation in the DG seem to be discrepant but it could be explained based on the viral expression pattern and recent paper dissecting the functions of dentate gyrus granule cells (Nakashiba et al., 2012) (Fig. 19). As viral mLLP overexpression seems to be limited to the granule cells but not proliferating cells, the increased cell proliferation seems to be non-cell-autonomous. Overexpressed mLLP might have indirectly enhanced the cell proliferation by affecting mature granule cells in DG. Increased activity of granule cells can facilitate the production of neurotrophic

factor such as BDNF or FGF that enhance the neurogenesis (Ma et al., 2009). Considering the effect in the cultured neurons, it could have enhanced the synaptic transmission and spine density *in vivo* as well. A recent report revealed that enhanced synaptic transmission of mature granule cells in DG promote pattern completion while young granule cells mediate pattern separation (Nakashiba et al., 2012). Therefore, mLLP overexpression might have biased the circuit to the pattern completion. The increased cell proliferation might be a result caused by the increased activity of mature granule cells. Cell type-specific manipulation and pattern completion experiments in the future would help to solve this problem.

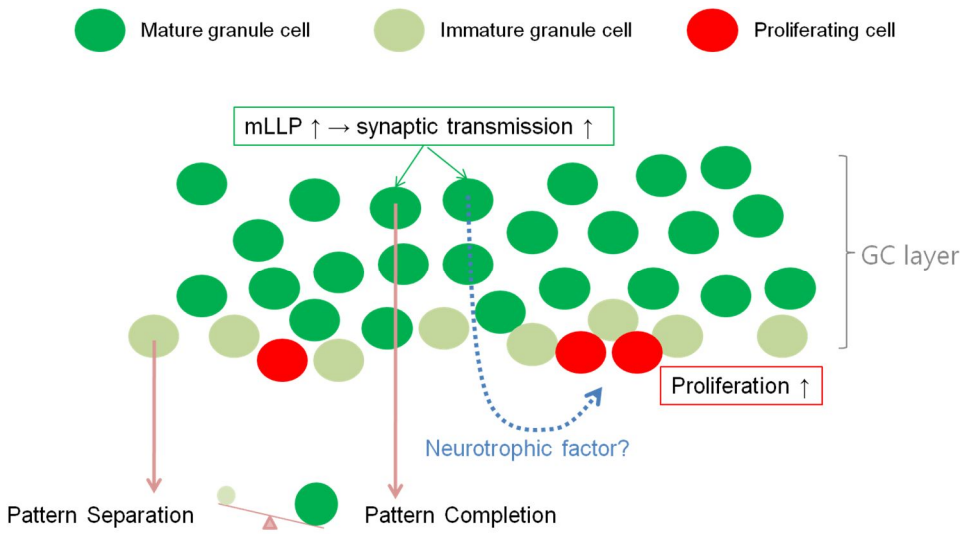


Figure 19. Hypothesis to explain the seemingly discrepant behavioral and immunohistochemistry results

As mLLP-overexpressing virus hardly infected the proliferating cells, it likely affected only the mature granule cells. If mLLP increased the synaptic transmission onto the granule cells in DG as in the neuron culture, it could have biased the circuit to the pattern completion, leading to impaired pattern separation. The increased cell proliferation might be the byproduct of this increased mature granule cell activity.

CHAPTER 3.

Molecular characteristics

and action mechanisms of mLLP

INTRODUCTION

Previous studies have found that ApLLP is a transcription factor enriched in the nucleus and nucleolus (Kim et al., 2003; Kim et al., 2006). The nuclear localization signals are in the N- and C-terminal arginine/lysine-rich positive charged regions. With N- and C-terminal conserved amino acid sequences, mLLP might also be localized to nucleus/nucleolus and function as a transcription regulator. In this chapter, I provide the evidences implicating the molecular function of mLLP.

Nucleolus is a subnuclear structure responsible for the ribosome biogenesis, therefore important for controlling the translation capacity and cellular growth (Emmott and Hiscox, 2009). In addition, it has been also reported that nucleolar proteins can regulate RNA polymerase II-regulated mRNA transcription (Louvet and Percipalle, 2008a). Since mLLP is a nucleolus-targeted protein, I assessed the possibilities whether mLLP could regulate the gross structure – number and size – of nucleoli or regulate transcription of mRNA as ApLLP did.

According to a recent high-throughput interactome data obtained by tandem affinity purification-mass spectrometry, human LLP (hLLP) interacts with CCCTC-binding factor (CTCF) (Hutchins et al., 2010). CTCF is a multifunctional 11-zinc-finger-containing and DNA-binding transcription factor acting as a transcriptional regulator, chromatin insulator, or mediator for the long-range chromatin interaction (Holwerda and de Laat, 2013; Phillips and Corces, 2009). Although CTCF is known to be a critical chromatin regulator, its role in the central nervous system has not been well studied. A recent study revealed that CTCF regulates transcription of ataxin-7 gene which is associated with spinocerebellar

ataxia type 7, a neurodegenerative disorder (Sopher et al., 2011b). Another important study found a de novo mutation on CTCF gene associated with intellectual disability (Gregor et al., 2013). In line with the clinical study, conditional knockout mouse with CTCF deletion in cortical and hippocampal neurons shows defects in dendritic arborization, spine formation, and synaptic transmission during postnatal development (Hirayama et al., 2012). As effects of CTCF deletion and mLLP reduction on the neuronal morphology or synaptic transmission are similar, I hypothesized that there might be a common downstream pathway of mLLP and CTCF.

As ApLLP is a member of intrinsically unstructured protein (IUP) family, the homologous LLP proteins might be also unstructured proteins. I checked this possibility by bioinformatic tool. Another possible characteristic that mLLP might have is the transducible property as many transducible proteins have the arginine-/lysine-rich sequences. To test this interesting possibility, I performed experiments to find the cellular effects of mLLP protein transduction.

EXPERIMENTAL PROCEDURES

DNA constructs

mLLP cDNA was inserted in frame into pEGFP-N1 vector to examine the subcellular localization of mLLP. pcDNA3.1(+)-3XFLAG was constructed by inserting 3XFLAG sequence from p3XFLAG-CMV-7.1 to tag in frame to the 3' end of mLLP or BDNF cds.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde / 4% sucrose in PBS. These cells were permeabilized with 0.1% Triton X-100, 0.1% BSA in PBS (PBT) and then blocked with 0.08% Triton X-100, 2% BSA in PBS. Antibodies were incubated in blocking solution. Primary antibodies (anti-fibrillarin(Covance), anti-GFP(Neuromab)) were incubated o/n at 4℃ and washed with PBT. Secondary antibodies conjugated with fluorescence dyes were incubated at room temperature for 2 hrs and washed with PBT. Samples were mounted on Vectashield with DAPI (VectorLab) and imaged using confocal microscopy (LSM700).

Co-immunoprecipitation

Epitope-tagged mLLP or vector control-transfected cells were lysed with hypotonic buffer (10 mM HEPES (pH7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) to isolate nuclei. Nuclei were lysed with lysis buffer (20 mM HEPES (pH7.9), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). Nuclear lysates were dialyzed with IP buffer (25 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton X-

100). Anti-His antibody (Santa Cruz) was incubated with the nuclear lysates o/n at 4℃ and then secondary antibody was incubated for 2 hrs. It was again incubated with protein G-coupled sepharose beads (GE) and washed with IP buffer with 0.1 mM EDTA and 0.2 % Triton X-100 and then buffer without Triton X-100. Immunoprecipitated proteins were eluted by 2X SDS sample buffer at 85℃ for 3 min, and subjected to immunoblotting. Antibodies used for immublotting were anti-nuclear myosin Iβ(Sigma), anti-actin, anti-RNA polymerase II(polII), anti-TATA-binding protein(TBP)(abcam), anti-CTCF(abcam).

Protein purification

pET21a-mLLP or pET21a-HA.GFP was transformed into BL21 E.coli. The E.coli was seeded in small volume LB with ampicillin and incubated in shaker o/n at 37℃. Next day, it was diluted in large volume LB and 0.5 mM IPTG was added at exponential growth phase. 2-4 hrs later, E.coli expressing mLLP or GFP were harvested and frozen until it was used for protein purification. E.coli was lyzed by sonication, and the lysate was centrifuged at 13000 rpm (SS-34) for 30 min. The supernatant was loaded onto the pre-charged and equilibrated His affinity column. After washing steps, protein was eluted with high imidazole elution buffer. Buffer was exchanged to PBS by size exclusion chromatography using PD-10 column. In case to obtain high concentration protein, purified proteins were subjected to methanol chloroform precipitation followed by dissolving in water, which was later diluted in PBS just before use.

Sholl analysis

Neurons were transfected with pcDNA3.1(+) or pcDNA3.1(+)-mLLP at 3 DIV. For visualization, GFP-expressing pSuper plasmid was co-transfected. Proteins were treated daily for 3 days. At 6 DIV, neurons were fixed with 4% paraformaldehyde / 4% sucrose in PBS. GFP images were taken under the fluorescence microscope and dendrites were traced using NeuronJ plugin of ImageJ. The traces were subjected to Sholl analysis using ImageJ Sholl analysis plugin. Protein-treated neurons were processed by the same method. Two or three independent cultures were used for each experiment.

RESULTS

Nuclear and nucleolar localization of mLLP

I assessed the subcellular localization of mLLP using a DNA construct that expresses mLLP fused to GFP. The plasmid was transfected to the dissociated primary neurons or NIH3T3 cell lines, both of which showed the typical localization pattern of mLLP localizing exclusively in the nucleus and more enriched in the subnuclear spots. Immunocytochemistry confirmed that the intense nuclear mLLP-GFP spots were mostly colocalized with fibrillarin, a nucleolar marker (Fig. 20). This shows that nuclear/nucleolar localization pattern is conserved in mLLP and ApLLP (Kim et al., 2003).

Effects of mLLP knockdown or overexpression on the nucleolus size and number

The function of nucleolus has been implicated in neurons during neuronal morphogenesis or stress response (Hetman and Pietrzak, 2012). As mLLP localizes to the nucleolus and is important for neuronal growth, I examined whether knockdown or overexpression of mLLP would alter the overall nucleolar structure. The AAV vectors expressing shRNA against mLLP or control scrambled shRNA were infected into the dissociated neuron culture together with shRNA-resistant mLLP expressing or control vector. Nucleoli were visualized by immunostaining of fibrillarin. Neither number (Fig. 21A) nor size of nucleoli (Fig. 21B) was changed by mLLP knockdown or overexpression (One way ANOVA, $p=0.7376$ for nucleolus number, $p=0.8306$ for nucleolus size).

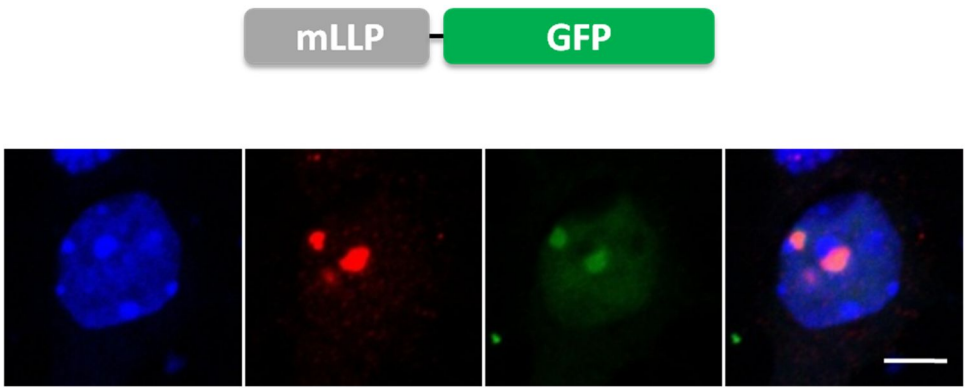


Figure 20. mLLP protein localizes to nucleus/nucleolus

Immunocytochemistry of neurons transfected with mLLP-GFP fusion construct using anti-fibrillarin antibody, a nucleolar marker. Scale Bar, 5 μm.

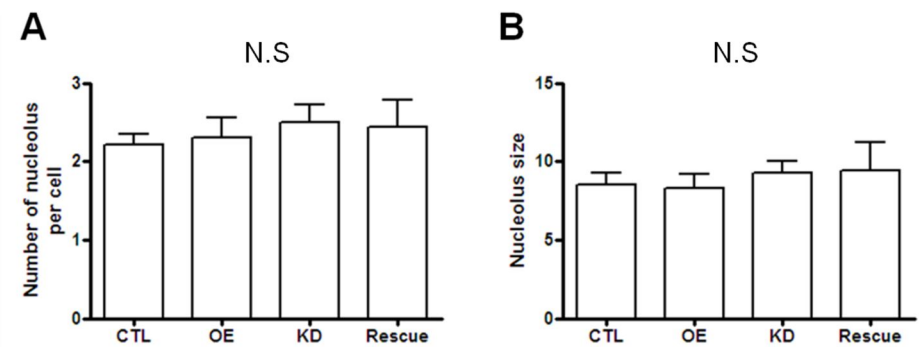


Figure 21. No gross change in nucleolar size and number

AAV expressing shRNA against mLLP or control shRNA were infected into the dissociated neuron culture with shRNA-resistant mLLP expressing or control vectors. Immunospots detected by fibrillarin antibody were analyzed using ImageJ. mLLP knockdown or overexpression did not affect neither number nor size of nucleoli (n=9-19 cells per group) *(in collaboration with Seohee Ahn).*

Co-immunoprecipitation assay reveals mLLP interacting proteins related with transcription

Next, in search for the interaction partners of mLLP in the nucleus, mass spectrometry following immunoprecipitation of overexpressed mLLP in NIH3T3 cell nuclear extract was performed and identified six candidate proteins to interact (Table 2). Co-immunoprecipitation experiment confirmed that mLLP interacted with nuclear myosin I β (NMIβ) and actin (Fig. 22). As nuclear myosin I and actin are involved in transcriptional regulation (Louvet and Percipalle, 2008b) and ApLLP acts as a transcription factor, immunoblotting was performed using antibodies against other transcription machineries such as RNA polymerase II (Pol II) and TATA-binding protein (TBP). They also interact with mLLP (Fig. 22). These results support that mLLP could act as a transcription regulator in complex with transcription machinery similarly to its homolog ApLLP.

Acc. No.	Protein name	Mol. wt. (kD)	pI	Sequence coverage (%)
23618899 M	Myosin VIIA	251.116	8.9	9
20137006 M	Myosin heavy chain IV	226.359	5.5	23
11067002	Nuclear myosin I beta	119.878	9.4	18
30316318 M	Zinc finger protein 64	71.766	8.8	18
49868 M	Beta-actin	39.186	5.8	25
12850774	Unnamed protein product	29.237	7	36

Table 2. Mass spectrometry of proteins co-immunoprecipitated with mLLP

(by Hyoung Kim)

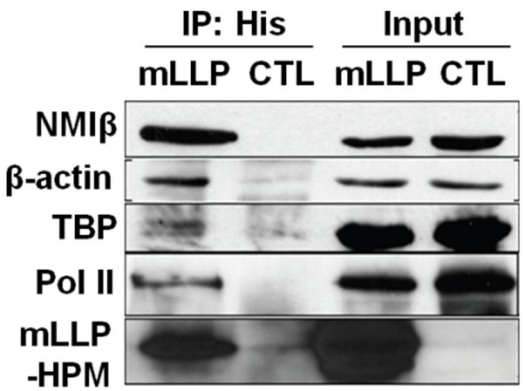


Figure 22. Coimmunoprecipitation of mLLP and transcriptional machinery proteins

Nuclear extracts from NIH3T3 cells trasnfected with hexahistidine-tagged mLLP or control vectors were immunoprecipitated with anti-histidine tag antibody. The immunoprecipitated products were immunoblotted with various antibodies against proteins related with transcription *(in collaboration with Hyoung Kim).*

mLLP interacts with CTCF

Interestingly, in a recent high throughput interactome study, human LLP (hLLP) was shown to interact with CTCF (Hutchins et al., 2010), that is critical for normal dendritic growth and spine formation in cortical and hippocampal neurons during postnatal development (Hirayama et al., 2012). To confirm the protein-protein interaction of mLLP and CTCF, the lysates of cells expressing 3XFLAG-tagged mLLP were immunoprecipitated with anti-FLAG IgG-conjugated agarose beads. Immunoblotting the eluates from the beads using anti-CTCF antibody detected the clear CTCF band (Fig. 23), indicating that LLP is an interaction partner of CTCF. As deficiency of both mLLP and CTCF lead to aberrant dendrite growth, spine density, and synaptic transmission, mLLP and CTCF might collaborate to regulate gene expression program important for neural development.

mLLP knockdown affects the expression of genes known to be regulated by CTCF

We examined whether mLLP regulates the expression of genes – APP (amyloid precursor protein) and protocadherin genes that have been reported to be under the control of CTCF in neurons and implicated for dendritic growth (Allinquant et al., 1995; Garrett et al., 2012; Lefebvre et al., 2012; Milward et al., 1992). Knockdown of mLLP by shRNA using AAV in dissociated hippocampal neuron culture from 0 DIV to 6 DIV downregulated the mRNA levels of APP (Fig. 24A) and Pcdhα12 (Fig. 24B), and Pcdhγa3 (Fig. 24C) were reduced supporting there is a common molecular pathway of mLLP and CTCF.

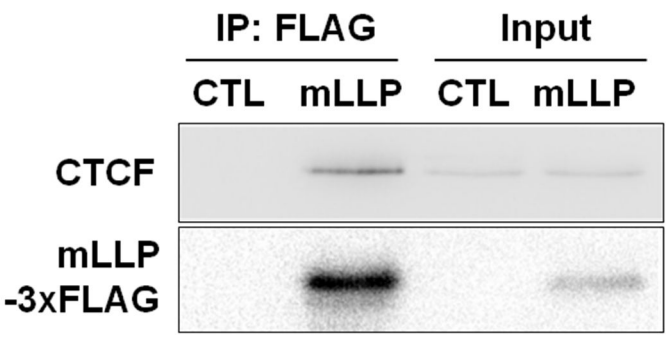


Figure 23. mLLP interacts with CTCF

mLLP-3XFLAG and CTCF in HEK293T nuclear lysates were coimmunoprecipitated by anti-FLAG antibody-conjugated agarose beads.

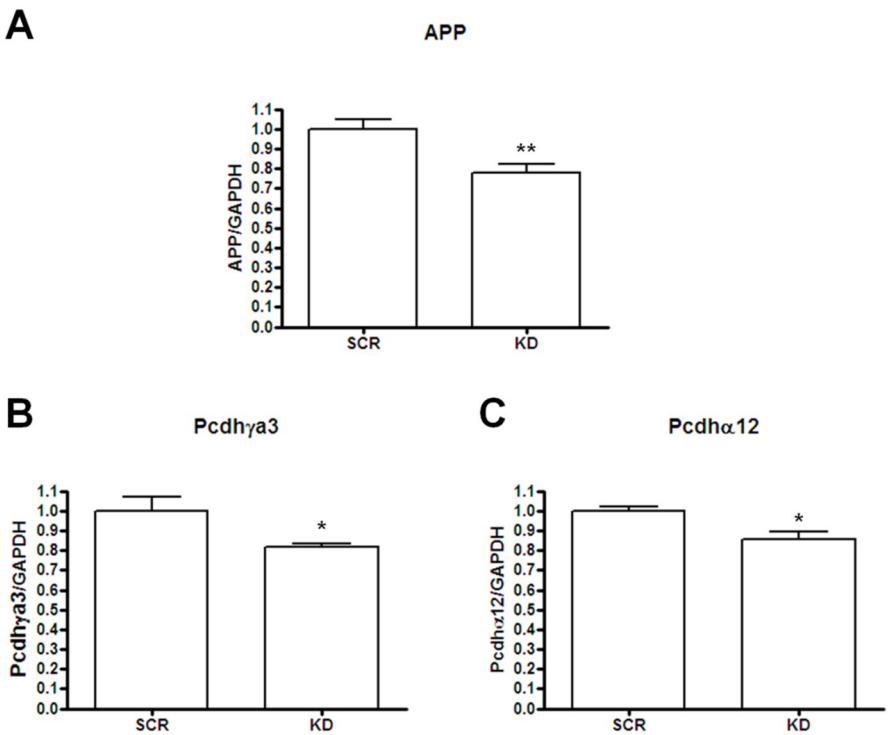
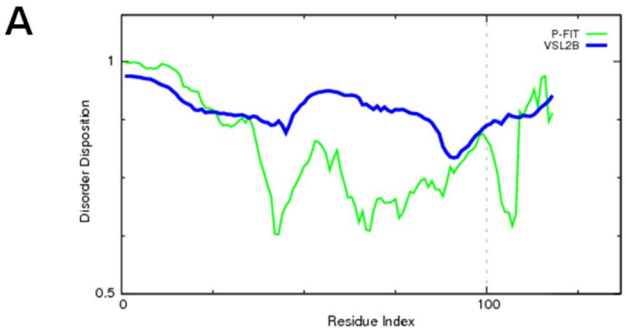


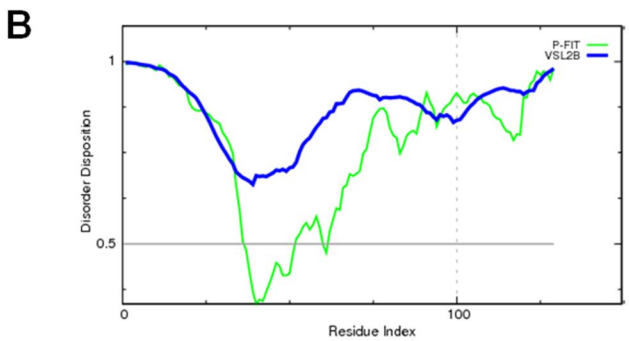
Figure 24. mLLP knockdown affects the expression of CTCF target genes

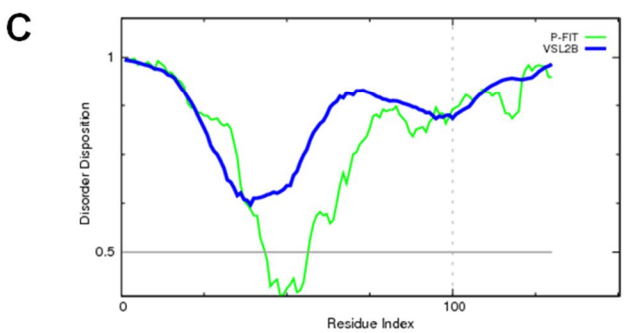
Quantitative RT-PCR after mLLP knockdown in dissociated hippocampal neuron culture from 0 DIV to 6 DIV using AAV. Unpaired t-test, **p<0.01, *p<0.05, n=4 per group *(in collaboration with Juyoun Yoo)*.

mLLP and hLLP are predicted to be intrinsically unstructured proteins

One interesting molecular feature of ApLLP is its disorderness. The proteins that do not have ordered structures so that their structure cannot be determined are called intrinsically unstructred protein (IUP). Using the protein disorder prediction tool DISPROT (http://www.disprot.org/metapredictor.php) used in the previous structural study on ApLLP (Liu and Song, 2008; Xue et al., 2010) (Fig. 25A), I found that mouse (Fig. 25C) and human (Fig. 25B) homologs of LLP are also predicted to be unstructured throughout entire amino acid sequences. Amino acid sequences of LLP proteins mostly have the score higher than 0.5 which is the threshold to predict the disorder probability. The disorder scores of mLLP and hLLP were slightly lower than ApLLP specifically in relatively hydrophobic 35-60 amino acid regions (Fig 25D). The hydrophobicity was scaled using the hydrophobicity calculator tool (http://web.expasy.org/protscale/). These data provide a clue to investigate the action mechanism of LLP proteins.


A
P-FIT
VSL2B
Disorder Disposition
1
0.5
0
100
Residue Index


B
P-FIT
VSL2B
Disorder Disposition
1
0.5
0
100
Residue Index


C
P-FIT
VSL2B
Disorder Disposition
1
0.5
0
100
Residue Index

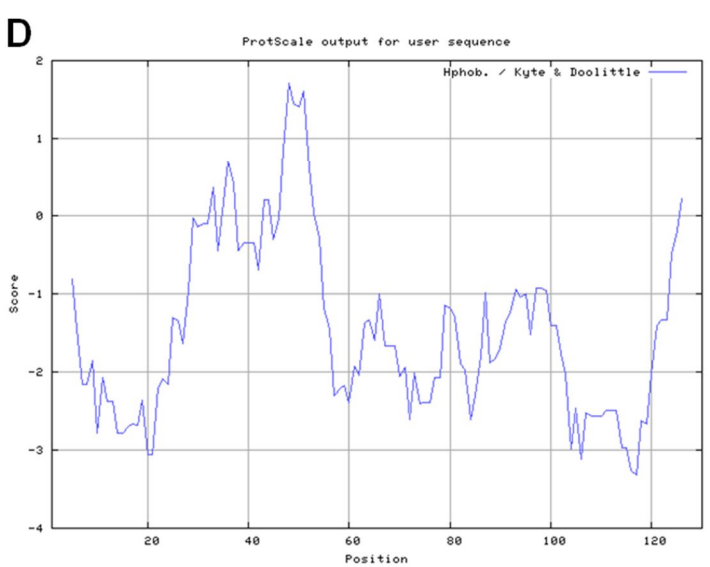

D
ProtScale output for user sequence
Hphob. / Kyte & Doolittle
Score
2
1
0
-1
-2
-3
-4
20
40
60
80
100
120
Position

Figure 25 . Amino acid analysis of LLPh proteins by bioinformatics

(A-C) Disorder analysis of ApLLP (A), hLLP (B), and mLLP (C) using VSL2B and PONDR-fit in DISPROT. (D) Hydrophobicity scales of mLLP amino acid sequences were calculated using ProtScale.

mLLP protein treated in the media enters the cells

As its N- and C-terminal regions of mLLP share the basic nature with most cell penetrating peptides (Futaki, 2005), these regions might act as protein transduction domains (PTD). First, it was tested whether mLLP protein can penetrate into cells. The hexahistidine-tagged mLLP protein was treated in the media of HEK293T cells, and cells were trypsinized to remove the proteins attached to the cell surface and harvested. The hexahistidine-tagged mLLP protein was detected in the cell lysates in the immunoblotting assays using the anti-hexahistidine tag antibody, indicating that mLLP protein has a transducible property (Fig. 26). Control GFP protein tagged with the hexahistidine epitope was not detected in the cell lysates. Moreover, hLLP protein, which has the amino acid sequence of 78% similarity to mLLP sequence, could also be internalized into cells (Fig. 27).

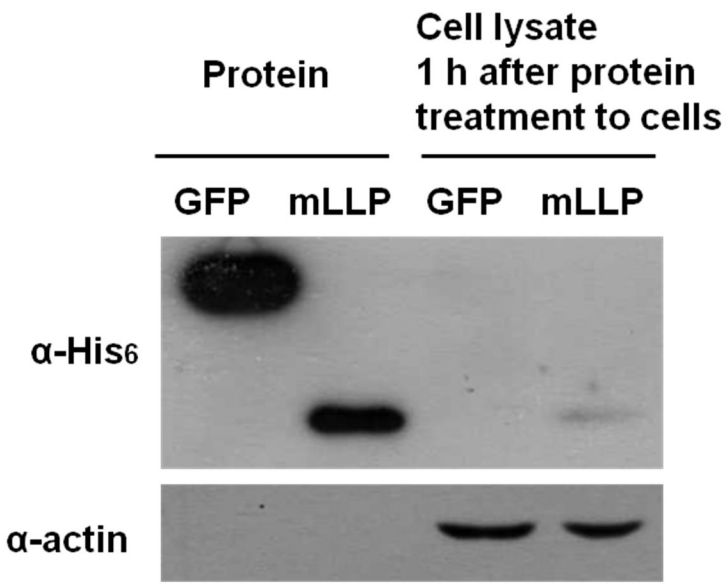


Figure 26. mLLP protein can be transduced into mammalian cells

Immunoblot analysis of HEK293T cell lysates 1 hr after mLLP or GFP protein treatment. Extracellularly treated mLLP-His6 protein was detected in the cell lysate while GFP was not detected.

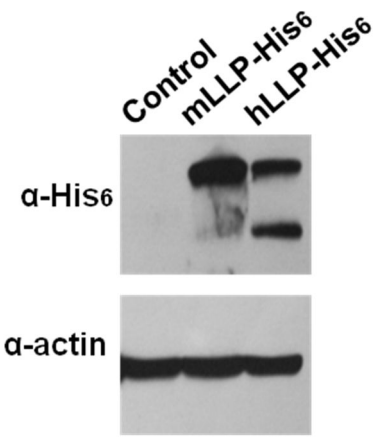


Figure 27. hLLP protein can be transduced into mammalian cells

hLLP protein treated in the media was detected in the cell lysates. hLLP protein purified from E.coli was partially cleaved but both intact and cleaved form could enter the cells *(in collaboration with Hyoung Kim).*

mLLP protein treatment enhances the dendrite growth

I examined whether treatment of mLLP protein to neurons affects maturation of neurons. After 3 days of daily protein treatment to the cultured neurons, the neurons were fixed and the GFP images were subjected to the Sholl analysis. Sholl analysis is a standard method to measure the degree of dendritic arborization by calculating the intersections of dendrites with the hypothetical circles with increasing radii from the soma. The Sholl analysis showed that mLLP protein treatment enhanced the dendritic arborization compared with the group treated with GFP protein (*p=0.0199, two-way ANOVA) (Fig. 28). The similar analysis was performed to compare the neurons that genetically overexpress mLLP and control neurons, which revealed the enhanced dendritic arborization in neurons overepxressing mLLP (Fig. 29). This indicates that this novel transducible protein has a capability of regulating neuronal growth when extracellularly treated, which is a similar effect of ectopic mLLP gene expression.

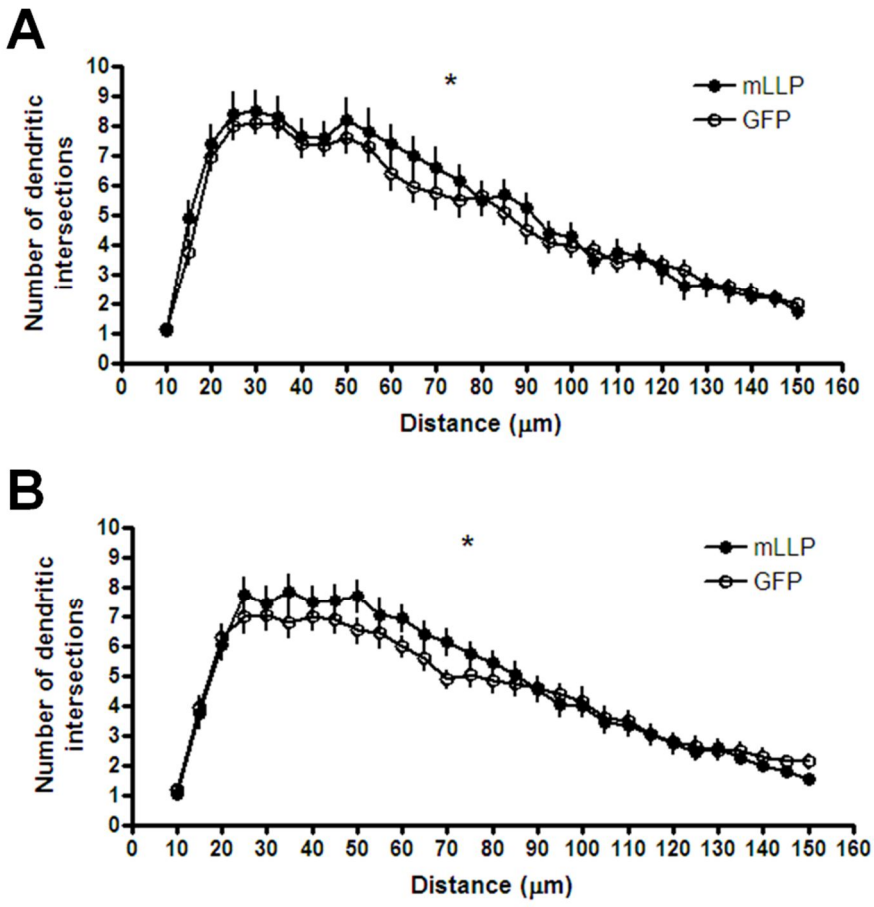


Figure 28. mLLP protein treatment increases dendritic arborization

(A) Sholl analysis after daily mLLP or GFP protein treatment (1μg/mL) for 3 days (n=29 cells for each group).

(B) As GFP and mLLP protein solution contained different salt concentration, protein treatment with matching the vehicle composition was also tested. (n=35 cells for each group, Two-way ANOVA, group effect , *p=0.0338)

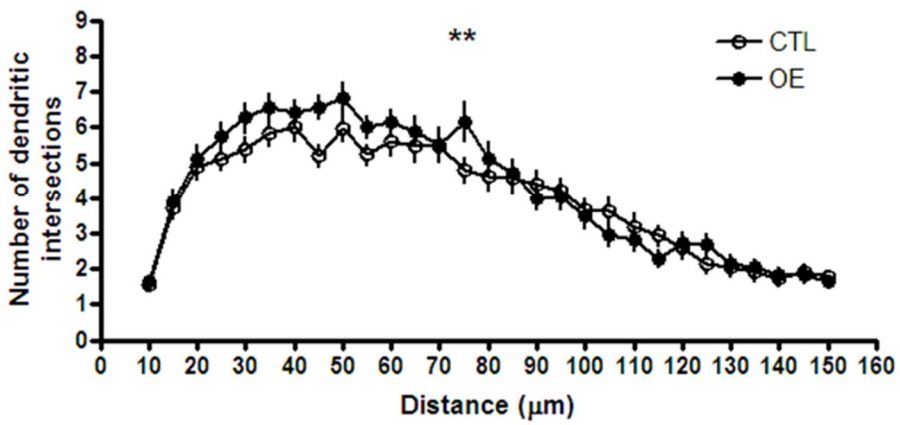


Figure 29. Genetic overexpression of mLLP enhances dendritic arborization

Sholl analysis 3 days after transfection of mLLP or control vector (n=30 cells for each group, Two-way ANOVA, group effect, **p=0.0048).

Low level of mLLP protein was detected in the extracellular media

LAPS18 was found to be a secretory protein although it does not have conventional secretory signal sequences (Nakaya et al., 2001). If mLLP is also a secretory protein, it might be transferred from one cell to another cell as a signaling molecule as it is capable of directly penetrating into the cells. Several transcription factors related with neurodevelopment have been found to have this property of transduction and unconventional secretion (Lesaffre et al., 2007; Prochiantz and Joliot, 2003; Sugiyama et al., 2008). To test whether mLLP might be one of them, the extracellular media of HEK293T cells transfected with plasmids overexpressing 3XFLAG-epitope-tagged mLLP was immunoprecipitated using anti-FLAG agarose beads (Fig. 30). As a positive control, 3XFLAG-tagged BDNF was expressed, and the media was immunoprecipitated with anti-FLAG beads, where I found strong bands in the media at the size of mature secretory form of BDNF. Relatively weak signals of mLLP were detected in the immunoprecipitates from the media of the mLLP-3XFLAG-overexpressing cells.

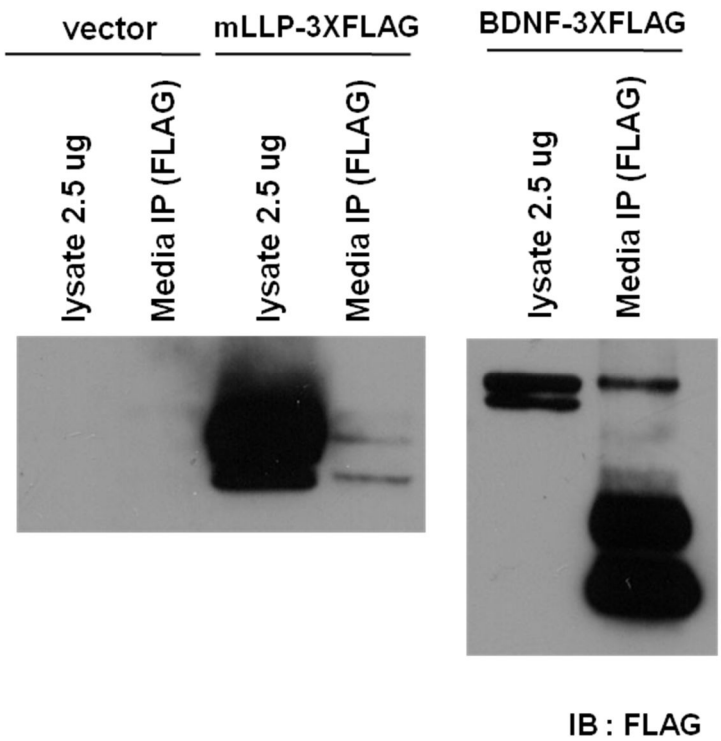


Figure 30. Small amount of mLLP was detected in the extracellular media of HEK293T cells overexpressing mLLP

Anti-FLAG immunoprecipitation of the supernatant media of 293T cells transfected with plasmids expressing mLLP-3XFLAG or BDNF-3XFLAG, or vector only. Secretory form of BDNF was strongly detected in the media. A small amount of mLLP was detected in the media

DISCUSSION

mLLP is a previously unknown nuclear/nucleolar protein characterized by its intrinsic disorder and transducibility. mLLP interacts with transcriptional regulators and CTCF. The lack of CTCF leads to the phenotypes in neural development (Hirayama et al., 2012) similar to mLLP knockdown. mLLP knockdown in neurons causes downregulation of some CTCF target genes related with dendritic growth.

I did not find any gross structural changes of nucleoli in neurons by mLLP knockdown. However, nucleolar rRNA transcription or other function could be altered with mLLP up or downregulation (Gomes et al., 2011). CTCF has been reported to be localized in nucleoli and represses nucleolar transcription (Torrano et al., 2006), which suggests the possibility that mLLP as a nucleolar protein and a CTCF binding partner, might also play a role for nucleolar transcription. Another candidate for the binding partner of LLP found in a high throughput interactome study is eIF2AK2 (eukaryotic translation initiation factor 2 alpha kinase 2) (Varjosalo et al., 2013) which has also been implicated in nucleolar proteome (Bański et al., 2010; Emmott and Hiscox, 2009). This aspect of molecular role of mLLP for nucleolar function needs to be assessed in the future.

Modification of 3D architecture of chromatin is an important part of transcriptional regulation. One of the proteins known to play a key role in this process is CTCF, the only documented major insulator-binding protein in vertebrates (Cuddapah et al., 2009; Ong and Corces, 2014). CTCF is a multifunctional DNA-binding zinc-finger protein, so it activates or represses transcription of various genes and mediates the long-range chromatin interaction

and organizes chromatin (Phillips and Corces, 2009). Recently, CTCF has been found to regulate neural development (Hirayama et al., 2012). Several genes that are important for neural development have been shown to be regulated by CTCF including BDNF (Chang et al., 2010), ataxin (Sopher et al., 2011a), APP (Vostrov et al., 2002; Yang et al., 1999), and protocadherins (Golan-Mashiach et al., 2012; Hirayama et al., 2012). Notably, CTCF deletion in the cortical and hippocampal projection neurons reduces dendritic arborization, spine density, and synaptic transmission (Hirayama et al., 2012). CTCF acts in protein complex, and a variety of its function comes from its interaction with various other proteins (Zlatanova and Caiafa, 2009). mLLP interacts with CTCF and both proteins contribute to neural development. The genes found here to be regulated by both proteins in common are some protocadherin genes and APP, which have been reported to be important for neurite outgrowth and arborization as well as dendritic spine structures (Garrett et al., 2012; Lefebvre et al., 2012; Mattson, 1994; Spires et al., 2005; Suo et al., 2012). This indicates that CTCF, at least in part, might work through the interaction with mLLP to regulate expression of some genes that are important for dendritic growth and spinogenesis. However, the detailed molecular mechanism of how mLLP would cooperates with CTCF needs to be explored.

It is notable that mLLP protein is transducible. Although mLLP is predominantly localized to the nucleus/nucleolus, it can also enter the cells from external environment, which is unconventional (Prochiantz, 2000). This suggests the possibility that mLLP proteins introduced from the extracellular space might also have a role for regulating neural development as other homeodomain transcription factors that are transducible and regulate neural development (Joliot and Prochiantz,

2004).

As LAPS18 is a secretory protein (Nakaya et al., 2001), mLLP might also be a secretory protein. However, since the level of mLLP detected in the media was very low compared to that of secreted BDNF and mLLP protein in the media might just have been released from dead cells, it is still unclear if mLLP is a secretory protein. Nevertheless, it cannot be excluded that the efficiency and the context of BDNF secretion might be different from mLLP. Therefore, to test the possibility that the release of mLLP into the extracellular space is a regulated process or not, more experiments should be performed. In addition, other cell types like neurons or glial cells in the context of development could be tested for the possibility of secretion of mLLP protein.

CHAPTER 4. CONCLUSION

CONCLUSION

This study is highlighting the unknown role of mouse homolog of LLP in the central nervous system. As I found that mLLP protein is highly expressed during the early developmental phase in the brain and neuron culture, I focused on the role of mLLP for the neural development. During the neuronal morphogenesis and synaptic maturation in the primary hippocampal neuron culture, mLLP knockdown led to abnormal dendritic morphology and reduced the synapse and spine density. It also decreased synaptic transmission measured by mEPSC amplitude. Conversely, mLLP overexpression enhanced the dendritic growth and increased the spine density. In line with the increased spine density, mEPSC frequency was increased by mLLP overexpression. This is the first report suggesting a role of LLP family protein for the neural development. To examine whether the brain development requires LLP *in vivo* and explore the more detailed mechanism and behavioral consequences, knockout mouse study will be useful in the future.

This study reports that mLLP localizes to the nucleus/nucleolus and interacts with transcriptional machinery, indicating its role in regulating transcription. mLLP also interacts with a transcription factor CTCF, which is similarly important for dendritic growth and spine formation (Hirayama et al., 2012). APP (Mattson, 1994; Spires et al., 2005) and some protocadherin genes (Garrett et al., 2012) that play a role in growth of neuritis and spines and known to be controlled also by CTCF (Golan-Mashiach et al., 2012; Vostrov et al., 2002) appeared to be downregulated by mLLP knockdown. This is the first report giving the clue for the molecular role of mLLP, which is largely unknown. Moreover, CTCF has been recently found to

be required for neural development (Hirayama et al., 2012), but the known molecular mechanism related with this process is limited. The present evidence could give a clue to discover unknown regulatory mechanism that involves both CTCF and mLLP.

Interestingly, mLLP protein has a unique property of transduction into cells. mLLP protein application could affect the dendritic growth in the same direction with genetic overexpression of mLLP, implying that this protein might be used for therapy or delivery of other proteins.

Furthermore, mLLP overexpression in the adult mouse brain altered mouse behavior in terms of context discrimination, which suggests that mLLP can modulate behavior *in vivo* possibly through regulating the synaptic density and transmission considering the effects in the culture.

Future studies will address the role of mLLP in regulating the mouse behavior by genetic overexpression or knockdown of mLLP or by injecting the mLLP protein utilizing its transducibility. Especially, the possibility to apply mLLP protein to enhance the growth of neuronal structure like dendrites or spines in disease models accompanying the structural destabilization of dendrites or spines will be assessed. In addition, the molecular action mechanism of mLLP should be explored much more in detail.

REFERENCES

Amini, M., Ma, C.L., Farazifard, R., Zhu, G., Zhang, Y., Vanderluit, J., Zoltewicz, J.S., Hage, F., Savitt, J.M., Lagace, D.C., *et al.* (2013). Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury. Journal of Neuroscience *33*, 5773-5784.

Bański, P., Kodiha, M., and Stochaj, U. (2010). Chaperones and multitasking proteins in the nucleolus: networking together for survival? Trends in Biochemical Sciences *35*, 361-367.

Bertoncini, C.W., Jung, Y.S., Fernandez, C.O., Hoyer, W., Griesinger, C., Jovin, T.M., and Zweckstetter, M. (2005). Release of long-range tertiary interactions potentiates aggregation of natively unstructured α-synuclein. Proceedings of the National Academy of Sciences of the United States of America *102*, 1430-1435.

Brumwell, C.L., and Curran, T. (2006). Developmental mouse brain gene expression maps. The Journal of physiology *575*, 343-346.

Brunet, I., Weinl, C., Piper, M., Trembleau, A., Volovitch, M., Harris, W., Prochiantz, A., and Holt, C. (2005). The transcription factor Engrailed-2 guides retinal axons. Nature *438*, 94-98.

Chang, J., Zhang, B., Heath, H., Galjart, N., Wang, X., and Milbrandt, J. (2010). Nicotinamide adenine dinucleotide (NAD)–regulated DNA methylation alters CCCTC-binding factor (CTCF)/cohesin binding and transcription at the BDNF locus. Proceedings of the National Academy of Sciences *107*, 21836-21841.

Choi, J.-H., Yu, N.-K., Baek, G.-C., Bakes, J., Seo, D., Nam, H.J., Baek, S.H., Lim,

C.-S., Lee, Y.-S., and Kaang, B.-K. (2014). Optimization of AAV expression cassettes to improve packaging capacity and transgene expression in neurons. Molecular brain *7*, 1-10.

Clelland, C.D., Choi, M., Romberg, C., Clemenson Jr, G.D., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., *et al.* (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science *325*, 210-213.

Cline, H.T. (2001). Dendritic arbor development and synaptogenesis. Current opinion in neurobiology *11*, 118-126.

Copolovici, D.M., Langel, K., Eriste, E., and Langel, Ü. (2014). Cell-Penetrating Peptides: Design, Synthesis, and Applications. ACS Nano *8*, 1972-1994.

Creer, D.J., Romberg, C., Saksida, L.M., Van Praag, H., and Bussey, T.J. (2010). Running enhances spatial pattern separation in mice. Proceedings of the National Academy of Sciences of the United States of America *107*, 2367-2372.

Cuddapah, S., Jothi, R., Schones, D.E., Roh, T.-Y., Cui, K., and Zhao, K. (2009). Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. Genome research *19*, 24-32.

Das, S., and Mukhopadhyay, D. (2011). Intrinsically unstructured proteins and neurodegenerative diseases: conformational promiscuity at its best. IUBMB life *63*, 478-488.

de la Torre-Ubieta, L., and Bonni, A. (2011). Transcriptional Regulation of Neuronal Polarity and Morphogenesis in the Mammalian Brain. Neuron *72*, 22-40.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: How does adult hippocampal neurogenesis affect learning and memory? Nature

Reviews Neuroscience *11*, 339-350.

Di Lullo, E., Haton, C., Le Poupon, C., Volovitch, M., Joliot, A., Thomas, J.-L., and Prochiantz, A. (2011). Paracrine Pax6 activity regulates oligodendrocyte precursor cell migration in the chick embryonic neural tube. Development *138*, 4991-5001.

Dyson, H.J. (2012). Roles of intrinsic disorder in protein–nucleic acid interactions. Molecular bioSystems *8*, 97-104.

Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nature Reviews Molecular Cell Biology *6*, 197-208.

Emmott, E., and Hiscox, J.A. (2009). Nucleolar targeting: the hub of the matter. EMBO reports *10*, 231-238.

Emoto, K. (2011). Dendrite remodeling in development and disease. Development Growth and Differentiation *53*, 277-286.

Fanselow, M.S., and Dong, H.-W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? Neuron *65*, 7-19.

Frankel, A., and Pabo, C. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. Cell *55*, 1189-1193.

Futaki, S. (2005). Membrane-permeable arginine-rich peptides and the translocation mechanisms. Advanced drug delivery reviews *57*, 547-558.

Fuxreiter, M. (2012). Fuzziness: linking regulation to protein dynamics. Molecular BioSystems *8*, 168-177.

Garrett, A.M., Schreiner, D., Lobas, M.A., and Weiner, J.A. (2012). γ-Protocadherins control cortical dendrite arborization by regulating the activity of a FAK/PKC/MARCKS signaling pathway. Neuron *74*, 269-276.

Golan-Mashiach, M., Grunspan, M., Emmanuel, R., Gibbs-Bar, L., Dikstein, R., and Shapiro, E. (2012). Identification of CTCF as a master regulator of the clustered protocadherin genes. Nucleic acids research *40*, 3378-3391.

Gomes, C., Smith, S.C., Youssef, M.N., Zheng, J.-J., Hagg, T., and Hetman, M. (2011). RNA polymerase 1-driven transcription as a mediator of BDNF-induced neurite outgrowth. Journal of biological chemistry *286*, 4357-4363.

Gregor, A., Oti, M., Kouwenhoven, Evelyn N., Hoyer, J., Sticht, H., Ekici, Arif B., Kjaergaard, S., Rauch, A., Stunnenberg, Hendrik G., Uebe, S., *et al.* (2013). De Novo Mutations in the Genome Organizer CTCF Cause Intellectual Disability. The American Journal of Human Genetics *93*, 124-131.

Grillon, C., Southwick, S.M., and Charney, D.S. (1996). The psychobiologioal basis of posttraumatic stress disorder. Molecular Psychiatry *1*, 278-297.

Gsponer, J., Futschik, M.E., Teichmann, S.A., and Babu, M.M. (2008). Tight regulation of unstructured proteins: From transcript synthesis to protein degradation. Science *322*, 1365-1368.

Harris, K.M. (1994). Dendritic spines. eLS.

Heitz, F., Morris, M.C., and Divita, G. (2009). Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. British journal of pharmacology *157*, 195-206.

Hetman, M., and Pietrzak, M. (2012). Emerging roles of the neuronal nucleolus. Trends in Neurosciences *35*, 305-314.

Hirayama, T., Tarusawa, E., Yoshimura, Y., Galjart, N., and Yagi, T. (2012). CTCF is required for neural development and stochastic expression of clustered Pcdh genes in neurons. Cell Reports.

Hitti, F.L., and Siegelbaum, S.A. (2014). The hippocampal CA2 region is essential for social memory. Nature *508*, 88-92.

Holwerda, S.J.B., and de Laat, W. (2013). CTCF: the protein, the binding partners, the binding sites and their chromatin loops. Philosophical Transactions of the Royal Society B: Biological Sciences *368*, 20120369.

Hutchins, J.R.A., Toyoda, Y., Hegemann, B., Poser, I., Hériché, J.K., Sykora, M.M., Augsburg, M., Hudecz, O., Buschhorn, B.A., and Bulkescher, J. (2010). Systematic analysis of human protein complexes identifies chromosome segregation proteins. Science *328*, 593-599.

Iakoucheva, L.M., Brown, C.J., Lawson, J.D., Obradović, Z., and Dunker, A.K. (2002). Intrinsic disorder in cell-signaling and cancer-associated proteins. Journal of Molecular Biology *323*, 573-584.

Joliot, A., and Prochiantz, A. (2004). Transduction peptides: from technology to physiology. Nature cell biology *6*, 189-196.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nature protocols *1*, 2406-2415.

Kandel, E.R., Schwartz, J.H., and Jessell, T.M. (2000). Principles of neural science, Vol 4 (McGraw-Hill New York).

Kim, H., Chang, D.J., Lee, J.A., Lee, Y.S., and Kaang, B.K. (2003). Identification of nuclear/nucleolar localization signal in< i> Aplysia</i> learning associated protein of slug with a molecular mass of 18 kDa homologous protein. Neuroscience letters *343*, 134-138.

Kim, H., Lee, S.H., Han, J.H., Lee, J.A., Cheang, Y.H., Chang, D.J., Lee, Y.S., and Kaang, B.K. (2006). A Nucleolar Protein ApLLP Induces ApC/EBP Expression

Required for Long-Term Synaptic Facilitation in< i> Aplysia</i> Neurons. Neuron *49*, 707-718.

Kitamura, T., Pignatelli, M., Suh, J., Kohara, K., Yoshiki, A., Abe, K., and Tonegawa, S. (2014). Island Cells Control Temporal Association Memory. Science (New York, NY).

Kulkarni, V.A., and Firestein, B.L. (2012). The dendritic tree and brain disorders. Molecular and Cellular Neuroscience *50*, 10-20.

Laeremans, A., Van de Plas, B., Clerens, S., Van den Bergh, G., Arckens, L., and Hu, T. (2013). Protein expression dynamics during postnatal mouse brain development. Journal of Experimental Neuroscience *7*, 1-14.

Langston, R.F., Stevenson, C.H., Wilson, C.L., Saunders, I., and Wood, E.R. (2010). The role of hippocampal subregions in memory for stimulus associations. Behavioural Brain Research *215*, 275-291.

Lefebvre, J.L., Kostadinov, D., Chen, W.V., Maniatis, T., and Sanes, J.R. (2012). Protocadherins mediate dendritic self-avoidance in the mammalian nervous system. Nature.

Lesaffre, B., Joliot, A., Prochiantz, A., and Volovitch, M. (2007). Direct non-cell autonomous Pax6 activity regulates eye development in the zebrafish. Neural development *2*, 2.

Leutgeb, J.K., Leutgeb, S., Moser, M.B., and Moser, E.I. (2007). Pattern separation in the dentate gyrus and CA3 of the hippocampus. Science *315*, 961-966.

Liu, J., Gaj, T., Patterson, J.T., Sirk, S.J., and Barbas III, C.F. (2014). Cell-Penetrating Peptide-Mediated Delivery of TALEN Proteins via Bioconjugation for Genome Engineering. PloS one *9*, e85755.

Liu, J., and Song, J. (2008). A novel nucleolar transcriptional activator ApLLP for long-term memory formation is intrinsically unstructured but functionally active. Biochemical and Biophysical Research Communications *366*, 585-591.

Louvet, E., and Percipalle, P. (2008a). Chapter 3 Transcriptional Control of Gene Expression by Actin and Myosin. In International Review of Cell and Molecular Biology, W.J. Kwang, ed. (Academic Press), pp. 107-147.

Louvet, E., and Percipalle, P. (2008b). Transcriptional control of gene expression by actin and myosin. International review of cell and molecular biology *272*, 107-147.

Ma, D.K., Jang, M.-H., Guo, J.U., Kitabatake, Y., Chang, M.-l., Pow-Anpongkul, N., Flavell, R.A., Lu, B., Ming, G.-l., and Song, H. (2009). Neuronal activity–induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science *323*, 1074-1077.

Mattson, M.P. (1994). Secreted forms of β-amyloid precursor protein modulate dendrite outgrowth and calcium responses to glutamate in cultured embryonic hippocampal neurons. Journal of neurobiology *25*, 439-450.

McHugh, T.J., Jones, M.W., Quinn, J.J., Balthasar, N., Coppari, R., Elmquist, J.K., Lowell, B.B., Fanselow, M.S., Wilson, M.A., and Tonegawa, S. (2007). Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. Science *317*, 94-99.

Nagahara, H., Vocero-Akbani, A., Snyder, E., Ho, A., Latham, D., Lissy, N., Becker-Hapak, M., Ezhevsky, S., and Dowdy, S. (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^ K^ i^ p^ 1 induces cell migration. Nature medicine *4*, 1998-1912.

Nakashiba, T., Cushman, J.D., Pelkey, K.A., Renaudineau, S., Buhl, D.L., McHugh, T.J., Barrera, V.R., Chittajallu, R., Iwamoto, K.S., and McBain, C.J. (2012). Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. Cell *149*, 188-201.

Nakaya, T., Kawahara, S., Watanabe, S., Lee, D.S., Suzuki, T., and Kirino, Y. (2001). Identification and expression of a novel gene in odour-taste associative learning in the terrestrial slug. Genes to Cells *6*, 43-56.

Nakazawa, K., McHugh, T.J., Wilson, M.A., and Tonegawa, S. (2004). NMDA receptors, place cells and hippocampal spatial memory. Nature Reviews Neuroscience *5*, 361-372.

Niibori, Y., Yu, T.-S., Epp, J.R., Akers, K.G., Josselyn, S.A., and Frankland, P.W. (2012). Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. Nature communications *3*, 1253.

Nimchinsky, E.A., Sabatini, B.L., and Svoboda, K. (2002). Structure and function of dendritic spines. Annu Rev Physiol *64*, 313-353.

O'Donnell, C., and Sejnowski, Terrence J. (2014). Selective Memory Generalization by Spatial Patterning of Protein Synthesis. Neuron *82*, 398-412.

Ong, C.-T., and Corces, V.G. (2014). CTCF: an architectural protein bridging genome topology and function. Nat Rev Genet *15*, 234-246.

Parikshak, N.N., Luo, R., Zhang, A., Won, H., Lowe, J.K., Chandran, V., Horvath, S., and Geschwind, D.H. (2013). Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. Cell *155*, 1008-1021.

Phillips, J.E., and Corces, V.G. (2009). CTCF: master weaver of the genome. Cell *137*, 1194-1211.

Phillips, R.G., and LeDoux, J.E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behavioral Neuroscience *106*, 274-285.

Prochiantz, A. (2000). Messenger proteins: homeoproteins, TAT and others. Current opinion in cell biology *12*, 400-406.

Prochiantz, A., and Joliot, A. (2003). Can transcription factors function as cell–cell signalling molecules? Nature Reviews Molecular Cell Biology *4*, 814-819.

Ramakrishna, S., Dad, A.-B.K., Beloor, J., Gopalappa, R., Lee, S.-K., and Kim, H. (2014). Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome research.

Raychaudhuri, S., Dey, S., Bhattacharyya, N.P., and Mukhopadhyay, D. (2009). The role of intrinsically unstructured proteins in neurodegenerative diseases. PLoS ONE *4*.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., and Hen, R. (2011a). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature *472*, 466-470.

Sahay, A., Wilson, D., and Hen, R. (2011b). Pattern Separation: A Common Function for New Neurons in Hippocampus and Olfactory Bulb. Neuron *70*, 582-588.

Sandhu, K.S. (2009). Intrinsic disorder explains diverse nuclear roles of chromatin remodeling proteins. Journal of Molecular Recognition *22*, 1-8.

Sopher, B.L., Ladd, P.D., Pineda, V.V., Libby, R.T., Sunkin, S.M., Hurley, J.B., Thienes, C.P., Gaasterland, T., Filippova, G.N., and La Spada, A.R. (2011a). CTCF

regulates ataxin-7 expression through promotion of a convergently transcribed, antisense noncoding RNA. Neuron *70*, 1071-1084.

Sopher, Bryce L., Ladd, Paula D., Pineda, Victor V., Libby, Randell T., Sunkin, Susan M., Hurley, James B., Thienes, Cortlandt P., Gaasterland, T., Filippova, Galina N., and La Spada, Albert R. (2011b). CTCF Regulates Ataxin-7 Expression through Promotion of a Convergently Transcribed, Antisense Noncoding RNA. Neuron *70*, 1071-1084.

Spatazza, J., Di Lullo, E., Joliot, A., Dupont, E., Moya, K.L., and Prochiantz, A. (2013a). Homeoprotein signaling in development, health, and disease: a shaking of dogmas offers challenges and promises from bench to bed. Pharmacological reviews *65*, 90-104.

Spatazza, J., Lee, H.H., Di Nardo, A.A., Tibaldi, L., Joliot, A., Hensch, T.K., and Prochiantz, A. (2013b). Choroid-plexus-derived otx2 homeoprotein constrains adult cortical plasticity. Cell reports *3*, 1815-1823.

Spires, T.L., Meyer-Luehmann, M., Stern, E.A., McLean, P.J., Skoch, J., Nguyen, P.T., Bacskai, B.J., and Hyman, B.T. (2005). Dendritic spine abnormalities in amyloid precursor protein transgenic mice demonstrated by gene transfer and intravital multiphoton microscopy. The Journal of neuroscience *25*, 7278-7287.

Sugiyama, S., Di Nardo, A.A., Aizawa, S., Matsuo, I., Volovitch, M., Prochiantz, A., and Hensch, T.K. (2008). Experience-Dependent Transfer of Otx2 Homeoprotein into the Visual Cortex Activates Postnatal Plasticity. Cell *134*, 508-520.

Suo, L., Lu, H., Ying, G., Capecchi, M.R., and Wu, Q. (2012). Protocadherin clusters and cell adhesion kinase regulate dendrite complexity through Rho GTPase.

Journal of molecular cell biology *4*, 362-376.

Tønnesen, J., Katona, G., Rózsa, B., and Nägerl, U.V. (2014). Spine neck plasticity regulates compartmentalization of synapses. Nature neuroscience *17*, 678-685.

Tau, G.Z., and Peterson, B.S. (2009). Normal development of brain circuits. Neuropsychopharmacology *35*, 147-168.

Torrano, V., Navascués, J., Docquier, F., Zhang, R., Burke, L.J., Chernukhin, I., Farrar, D., León, J., Berciano, M.T., and Renkawitz, R. (2006). Targeting of CTCF to the nucleolus inhibits nucleolar transcription through a poly (ADP-ribosyl) ation-dependent mechanism. Journal of cell science *119*, 1746-1759.

Varjosalo, M., Sacco, R., Stukalov, A., van Drogen, A., Planyavsky, M., Hauri, S., Aebersold, R., Bennett, K.L., Colinge, J., and Gstaiger, M. (2013). Interlaboratory reproducibility of large-scale human protein-complex analysis by standardized AP-MS. Nature methods *10*, 307-314.

Vostrov, A.A., Taheny, M.J., and Quitschke, W.W. (2002). A region to the N-terminal side of the CTCF zinc finger domain is essential for activating transcription from the amyloid precursor protein promoter. Journal of Biological Chemistry *277*, 1619-1627.

Wang, F., Wang, Y., Zhang, X., Zhang, W., Guo, S., and Jin, F. (2014). Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. Journal of Controlled Release *174*, 126-136.

Witter, M.P. (2009). Hippocampal formation. In Encyclopedia of Neuroscience (Springer), pp. 1840-1845.

Xue, B., Dunbrack, R.L., Williams, R.W., Dunker, A.K., and Uversky, V.N. (2010). PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. Biochimica

et Biophysica Acta (BBA)-Proteins and Proteomics *1804*, 996-1010.

Yang, Y., Quitschke, W., Vostrov, A.A., and Brewer, G.J. (1999). CTCF Is Essential for Up-Regulating Expression from the Amyloid Precursor Protein Promoter During Differentiation of Primary Hippocampal Neurons. Journal of neurochemistry *73*, 2286-2298.

Yassa, M.A., and Stark, C.E.L. (2011). Pattern separation in the hippocampus. Trends in Neurosciences *34*, 515-525.

Ye, B., Kim, J.H., Yang, L., McLachlan, I., Younger, S., Jan, L.Y., and Jan, Y.N. (2011). Differential regulation of dendritic and axonal development by the novel Krüppel-like factor Dar1. The Journal of Neuroscience *31*, 3309-3319.

Zadran, S., Bi, X., and Baudry, M. (2010). Regulation of calpain-2 in neurons: Implications for synaptic plasticity. Molecular Neurobiology *42*, 143-150.

Zhang, L.I., and Poo, M.-m. (2001). Electrical activity and development of neural circuits. Nature Neuroscience *4*, 1207-1214.

Zlatanova, J., and Caiafa, P. (2009). CTCF and its protein partners: divide and rule? Journal of cell science *122*, 1275-1284.

국문초록

신경발달 중 역동적으로 일어나는 뉴런의 형태 형성을 위해서는 여러 단계의 전사 프로그램의 복잡한 조절이 잘 이루어져야 한다. 정상적인 뉴런의 성숙을 위해서 어떻게 유전자 발현이 조절되는지의 전체적인 그림이 그려지기 위해서는 아직도 밝혀져야 하는 부분들이 많다. 본 연구에서는 선행 연구에서 군소 뉴런의 시냅스 가소성을 증진시키는 전사인자로 밝혀졌었던 ApLLP의 생쥐 상동단백질인 mLLP라는 새로운 핵 단백질이 뉴런 성숙에서 중요한 역할을 한다는 것을 새롭게 밝혀냈다.

뇌와 배양 뉴런에서 mLLP의 단백질 발현은 가장 어린 단계에서 높았고 발달이 진행되면서 확연히 감소하는 패턴을 보였다. 배양된 뉴런의 발달기에 shRNA를 이용하여 mLLP 발현양을 감소시키면 뉴런의 수상돌기 발달이 비정상적으로 변하였고 수상돌기가시의 밀도가 줄어들었으며, 이를 반영하듯 시냅스 전달 강도도 줄어들었다. 반대로 이 시기에 mLLP를 과발현시키면 반대 현상이 일어났다. 즉, 수상돌기 발달이 더 많이 일어나고, 수상돌기가시의 밀도는 더 높아졌으며, 시냅스 전달 강도 또한 강해졌다. 이과 같은 결과들은 mLLP가 이전에 보고된 바 없는 새로운 신경 발달에 관련된 단백질임을 보여주고 있다.

발생과정에 중요한 분자메커니즘이 성체가 된 이후에도 시냅스 가소성 및 학습에도 중요한 경우들이 많이 있다. 발생과정에서 중요한 mLLP 또한 행동에 어떤 영향이 있는지를 보기 위하여 mLLP를 생쥐 해마에서

과발현시킨 후 행동의 변화를 측정하였다. 생쥐 해마에서의 mLLP 과발현은 맥락적 공포조건화 기억 형성에는 영향을 주지 않았으나 그 이후에 맥락을 구분하는 학습이 저해되는 현상을 관찰하였다. 또한 DG에서의 신경세포발생은 증가하였다.

세포 내에서 mLLP는 핵과 인에 많은 양이 위치한다. 핵 추출물에서 mLLP 단백질은 전사 조절과 관련된 여러 단백질들과 상호작용하는 것으로 나타났다. 특히, 최근에 신경 발달에 중요하다고 밝혀진 바 있는 다기능 전사인자인 CTCF 단백질과 상호작용하는 것으로 나타났다. 또한 몇몇 CTCF 하위 유전자들의 발현양도 mLLP 감소에 의해서 줄어드는 것으로 나타났다.

흥미롭게도, mLLP 단백질은 세포 밖에 처리하였을 때 세포 안으로 들어갈 수 있는 성질이 있었다. 이와 같은 성질을 이용하여, 이 단백질을 직접 뉴런 세포 외부에 처리해주었을 때 mLLP를 유전자로 과발현했을 때와 비슷하게 수상돌기 발달을 증가시키는 경향이 나타났다. 인간의 상동유전자인 hLLP 단백질 또한 비슷하게 세포 내로 들어갈 수 있는 특징이 있다.

.....

주요어: LLP, 해마, 신경 발생, CTCF, PTD (protein transduction domain)